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SECTION MEETINGS

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| **CLEVELAND** | |
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### 10222

## Photodynamic Action of Safranine on Gram-Negative Bacilli.

T. T'UNG. (Introduced by S. H. Zia.)

*From the Department of Bacteriology and Immunology, Peiping Union Medical College, Peking.*

In a previous study on the photodynamic action of various dyes on bacteria,[1] it was found that several dyes were lethal for gram-positive organisms but none was effective for gram-negative bacilli. Efforts have, therefore, been continued to determine the photo-sensitivity of gram-negative organisms with dyes hitherto not used. After many trials, it was found that safranine was suitable for this purpose, and in the present communication, the result of an investigation of its photodynamic action on some common gram-negative bacilli is presented.

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[1] T'ung, T., and Zia, S. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 326.

Saturated aqueous solution of safranine* was diluted to different concentrations with distilled water, the pH of which was adjusted to 8.0 by the addition of sodium hydroxide. Organisms grown on plain meat-infusion agar-slants for 24 hours were suspended in water of the same pH and the number of bacteria was adjusted to about 500 millions per cc. The technic previously employed[1] for mixing the dye and organisms and for exposing to light was followed. Both exposed and unexposed mixtures were plated on China-blue rosalic-acid medium and incubated for 48 hours. The maximal photodynamic action of safranine on most of the gram-negative organisms could not be obtained without prolonged exposure; the results of a 2-hour exposure are presented in Table I.

The effects of the dye on unexposed bacteria have been omitted from the table because it was found that safranine possesses no native bactericidal power in 2 hours' contact with all gram-negative bacilli tested, except for *S. dysenteriæ* at 1-2 and for *V. choleræ* and *B. alcaligenes* at 1-100 dilution. From the table it is clear that safranine has marked photosensitizing power on a large number of varieties of gram-negative bacilli but it varies even among closely related species. It was further noted that a prozone in the photodynamic action of safranine was present on a majority of these organisms even after prolonged exposure. It seems that the prozone is more marked in cases in which the organisms are less susceptible to the photodynamic action. It appears also that the presence of a prozone is a group-characteristic. For instance, marked prozone reaction was observed in 15 strains of *E. coli,* but not in 15 strains of *K. pneumoniæ.*

Because of our previous experience[2] with the enhancing power of hydrogen peroxide on the photosensitizing activity of eosin on gram-positive organisms, it was considered of interest to see whether or not the photodynamic action of safranine on gram-negative ones could be increased by the addition of same reagent. Since *E. coli* is less susceptible to the photosensitization of this dye and comparatively more resistant to hydrogen peroxide, it alone was chosen for bearing out this fact. It was found that while safranine in the dilution of 1-1000 failed to kill *E. coli* after 60 minutes of exposure

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* Water-soluble safranine O made by the National Aniline and Chemical Company, U. S. A.

[2] T'ung, T., *Trans. X Congress, Far East. Assn. Trop. Med.,* in press.

TABLE I.
Growth in Exposed Mixtures of Bacteria and Safranine.

| Bacteria | Final concentrations of the dye | | | | | |
|---|---|---|---|---|---|---|
| | 1-2 | 1-10 | 1-10$^{2}$ | 1-10$^{3}$ | 1-10$^{4}$ | 1-10$^{5}$ |
| *V. choleræ* | — | — | — | — | — | ++++ |
| *B. alcaligenes* | — | — | — | — | ++ | ++++ |
| *Shigella* | | | | | | |
| *amigua* | — | — | — | — | — | ++++ |
| *paradysen. F* | — | — | — | — | — | ++++ |
| *paradysen. Y* | — | — | — | — | ++ | ++++ |
| *dysenteriæ* | — | — | — | — | ++ | ++++ |
| *E. typhi* | | | | | | |
| "O" (901) | — | — | — | — | ++++ | |
| "H" | — | — | — | — | ++++ | |
| "Watson" | — | — | — | ++ | ++++ | |
| *Prot.* | | | | | | |
| "O" (X-19) | — | — | — | — | ++++ | |
| "H" | — | — | — | ++++ | ++++ | |
| *Salmonella* | | | | | | |
| *paratyphi A* | + | — | — | — | ++++ | |
| *schottmuelleri* | ++ | — | — | + | ++++ | |
| *hirschfeldii* | +++ | ++ | — | ++ | ++++ | |
| *enteritidis* | ++ | — | — | — | ++++ | |
| *suipestifer* | +++ | ++ | — | +++ | ++++ | |
| *aertrycke* | +++ | ++ | — | +++ | ++++ | |
| *K. pneumoniæ* (S) | — | — | — | — | ++++ | |
| *K. pneumoniæ* (R) | — | — | — | — | ++++ | |
| *E. coli* | ++++ | +++ | — | — | ++++ | |
| *P. aeruginosa* | +++ | + | — | + | ++++ | |
| *B. prodigiosus* | ++++ | — | — | ++ | ++++ | |

— = no growth; + = growth less than 5 colonies; ++ = growth less than 50 colonies; +++ = growth less than 100 colonies; ++++ = growth more than 100 colonies.

to light, the addition of hydrogen peroxide in a final concentration of 0.15% enhanced the photosensitizing power and inactivated this organism after the same length of exposure. Safranine together with hydrogen peroxide in the absence of light or hydrogen peroxide alone in the presence of light had no bactericidal effect. Moreover, *E. coli* suspended in the above-mentioned concentration of hydrogen peroxide for one hour, washed and resuspended in water and then exposed to light with the dye for the same length of time was not killed. The result of the reënforcement of an oxidizing reagent with safranine in photosensitizing this organism suggests that oxidation may play a rôle in photodynamic action.

*Conclusion.* Safranine was found to be an efficient photosensitizing dye on certain gram-negative organisms. Its effect is characterized by the low native bactericidal power and the frequent occurrence of the prozonal phenomenon. The addition of hydrogen peroxide to the dye-organism mixture enhances the photosensitizing activity of the dye.

### 10223

## Solustibosan and Ureastibamine in Treatment of Kala-Azar in Chinese Hamsters.

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In a previous communication[1] it was demonstrated that Chinese hamsters infected with *Leishmania donovani* can be cured of the infection by neostibosan.

This report deals with chemotherapeutic tests with solustibosan and ureastibamine in Chinese hamsters with especial reference to their relative toxicity and curative value in comparison with neostibosan. Ureastibamine is known to be highly potent as well as highly toxic in the treatment of human kala-azar,[2] while solustibosan, a liquid preparation, which has the advantage of being administered intramuscularly, has been claimed to possess curative value and great tolerability in experimental and human kala-azar.[3]

*Experiment I.* Determination of the *maximum non-lethal dose* and *universal lethal* dose of solustibosan and ureastibamine in normal Chinese hamsters. The definition of doses and the experimental procedures, as described in a previous communication[4] were followed, with the exception that for each test dose a group of 10, instead of 20 animals was used. Ureastibamine was freshly prepared each time in a 5% sterile aqueous solution, and given subcutaneously. Solustibosan was used undiluted and also given subcutaneously. The results of the test doses are given in Tables I and II.

TABLE I.
Mortality Rate of Hamsters to Test Doses of Solustibosan.

| Dose, cc per kilo | No. of hamsters | No. of deaths | Mortality % |
|---|---|---|---|
| 20 | 10 | 0 | 0 |
| 30 | 10 | 0 | 0 |
| 40 | 10 | 2 | 20 |
| 80 | 10 | 4 | 40 |
| 100 | 10 | 4 | 40 |
| 120 | 10 | 9 | 90 |
| 140 | 10 | 10 | 100 |

From these tables it is evident that for solustibosan 30 cc per kilo is the *maximum non-lethal dose* and 140 cc per kilo, the *universal*

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[1] Wang, C. W., and Lee, C. U., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 674.

[2] Lee, C. U., and Chu, C. F., *Chinese Med. J.*, 1935, **49**, 328.

[3] Kikuth, W., and Schmidt, H., *Arch. Schiffs. u. Tropen-Hyg.*, 1938, **42**, 189.

[4] Wang, C. W., and Lee, C. U., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 670.

TABLE II.
Mortality Rate of Hamsters to Test Doses of Ureastibamine.

| Dose, g per kilo | No. of hamsters | No. of deaths | Mortality % |
|---|---|---|---|
| 0.5 | 10 | 0 | 0 |
| 1.0 | 10 | 2 | 20 |
| 2.0 | 10 | 5 | 50 |
| 3.0 | 10 | 10 | 100 |

*lethal dose;* and for ureastibamine 0.5 g and 3.0 g per kilo respectively. As the expression of the units of doses for solustibosan, ureastibamine and neostibosan was not the same the comparison between the different doses could only be made with their respective antimony contents. It is known that 1 cc of solustibosan contains 20 mg of Sb; 100 mg of neostibosan, 42 mg of Sb; and 100 mg of ureastibamine, 35 mg of Sb. The value of the antimony content in ureastibamine was based on Brahmarchari's determination, but it has been found to vary from 20 to 43% in different samples.[5] In Table III the different doses with their antimony equivalents are given, and the doses for neostibosan were taken from a previous work.[4]

TABLE III.
Antimony Equivalents in ***Maximum Non-lethal Dose*** and ***Universal Lethal Dose*** of Solustibosan, Neostibosan, and Ureastibamine in Normal Hamsters.

| | Maximum non-lethal dose | | | Universal lethal dose | | |
|---|---|---|---|---|---|---|
| | cc per kilo | g per kilo | mg Sb per kilo | cc per kilo | g per kilo | mg Sb per kilo |
| Solustibosan | 30 | | 600 | 140 | | 2800 |
| Neostibosan | | 1 | 420 | | 4 | 1680 |
| Ureastibamine | | 0.5 | 175 | | 3 | 1050 |

From Table III it is obvious that the antimony contents of the *maximum non-lethal dose* and *universal lethal dose* are highest in solustibosan and lowest in ureastibamine. This indicates that hamsters can tolerate much more antimony in the form of solustibosan than in the form of either neostibosan or ureastibamine. In other words, among the 3 antimony compounds tested, solustibosan is least and ureastibamine most toxic for normal hamsters.

*Experiment II.* The relative curative value of solustibosan, neostibosan, and ureastibamine in infected Chinese hamsters. Sixty infected hamsters were equally divided into 3 groups, each consisting of 20 hamsters. Each group was treated exclusively with one drug.

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[5] Ghosh, S., Chopra, R. N., and Chatterjee, N. R., *Ind. J. Med. Res.*, 1928, **16**, 461.

Treatment was started 77 days after the infection with kala-azar. Neostibosan was freshly prepared each time in a 1% sterile aqueous solution and ureastibamine in a 0.5% solution. A constant dose of 400 mg per kilo for neostibosan and 200 mg per kilo for ureastibamine was given 3 times a week subcutaneously. Solustibosan was given undiluted and also subcutaneously. A constant dose of 8 cc per kilo was given in the same frequency as the other 2, until a total of 120 cc per kilo was reached, when a constant dose of 16 cc per kilo was maintained. One to 4 hamsters were killed from time to time during the course of treatment and smears of spleen, liver and bone marrow were examined for parasites. The results are given in Table IV in which only the parasitological findings of spleen smears were recorded, as they were found to be most conclusive.

TABLE IV.

Relative Value of Solustibosan, Neostibosan, and Ureastibamine in Experimental Kala-azar in Chinese Hamsters.

| Total dose per kilo in | | | No. hamsters killed and examined | Leishman-donovan bodies in spleen | | Mortality during treatment, % |
|---|---|---|---|---|---|---|
| cc | g | mg Sb | | No. positive hamsters | No. negative hamsters | |
| | | | Solustibosan | | | |
| 24 | | 480 | 4 | 4 | 0 | |
| 48 | | 960 | 2* | 2 | 0 | |
| 72 | | 1440 | 2 | 2 | 0 | |
| 96 | | 1920 | 4 | 4 | 0 | |
| 120 | | 2400 | 3 | 2 | 1 | 10 |
| 152 | | 3040 | 3 | 0 | 3 | |
| 200 | | 4000 | 2 | 0 | 2 | |
| | | | Neostibosan | | | |
| | 1.2 | 504 | 4 | 4 | 0 | |
| | 2.4 | 1008 | 2 | 2 | 0 | |
| | 3.6 | 1512 | 2 | 0 | 2 | |
| | 4.8 | 2016 | 2 | 1 | 1 | 30 |
| | 6.0 | 2520 | 2 | 0 | 2 | |
| | 7.6 | 3192 | 2 | 0 | 2 | |
| | | | Ureastibamine | | | |
| | 1.2 | 420 | 4 | 4 | 0 | |
| | 2.2 | 770 | 3 | 2 | 1 | 60 |
| | 2.6 | 910 | 1 | 0 | 1 | |

*These two hamsters only received liver puncture and the treatment in them was continued.

In Table IV it is shown that negative spleen smears in the treated hamsters began to appear after a total of 120 cc or 2400 mg Sb per kilo in the form of solustibosan, 3.6 g or 1512 mg Sb per kilo in the form of neostibosan and only 2.2 g or 770 mg Sb per kilo in the form of ureastibamine. It is obvious that much more antimony in the form of solustibosan is required to give the same curative effect as with either neostibosan or ureastibamine. In terms of their antimony contents neostibosan is certainly a more effective drug than solustibosan, but inferior to ureastibamine in infected hamsters (the same conclusion is true when neostibosan and ureastibamine are compared by weight).

The mortality rate during the course of treatment was highest in the group of hamsters treated with ureastibamine, and lowest with solustibosan. The high mortality rate with ureastibamine probably can partly be explained by the occurrence of extensive ulceration and infection at the site of injection, as a result of improper administration of the drug by subcutaneous route, and as the intravenous injections in hamsters can not be carried out (solustibosan, however, gave practically no local reaction to hamsters). Nevertheless, the difference of mortality rate between solustibosan and neostibosan and between neostibosan and ureastibamine is not statistically significant.

*Summary and Conclusions.* The *maximum non-lethal dose* and *universal lethal dose* of solustibosan and ureastibamine in normal Chinese hamsters were determined and their values were compared with those neostibosan. In terms of their antimony contents hamsters tolerated much more antimony in the form of solustibosan than in the form of either neostibosan or ureastibamine. Evidently solustibosan is least, while ureastibamine is most toxic among the 3 antimony compounds for normal hamsters.

In the infected hamsters much more antimony in the form of solustibosan than that in the form of either neostibosan or ureastibamine was required to bring about a cure. It is obvious that ureastibamine is the most and solustibosan the least potent drug in the treatment of infected hamsters. The mortality rate during the course of treatment was highest with ureastibamine and lowest with solustibosan, but the differences are not statistically significant.

## 10224

## Polysaccharides of *C. diphtheriæ.*

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Recent serological studies[1, 2, 3] of *C. diphtheriæ* have shown that in addition to the 3 main types, *gravis,* intermediate, and *mitis,* there are many sub-types. Since polysaccharides have been accepted generally to be responsible for the type-specificity of many kinds of bacteria it was considered of interest to determine whether or not polysaccharides determine the antigenic types of *C. diphtheriæ.*

With the exception of one intermediate strain, Park 8, all of the organisms used in this study were isolated locally. All conformed to the cultural characteristics as set forth by Anderson and his associates[4] for *gravis,* intermediate, and *mitis.* One organism from each of the 3 cultural types and one avirulent strain were grown on stomach-digest medium[5] for a week. The methods of preparing the polysaccharides were essentially the same as reported for *B. rhinoscleromatis*[6] except that the extraction-time was one hour instead of half an hour, and 1% potassium hydroxide instead of 0.5% was used in preparing the alkali-fraction. The polysaccharides thus obtained were light brown powders, easily soluble in concentration of 1% and dilute solutions gave a strong Molisch reaction. The total yields were so small that detailed chemical characterization of these polysaccharides can not be made at this time.

Antisera were prepared with heat-killed cultures of 2 strains of each type. Two rabbits for each strain received 3 daily intravenous injections followed by 5 days of rest; this course was repeated twice. Seven days after the last injection serum was obtained and tested with the homologous polysaccharide. Only those sera with a high titer were tested for cross-precipitation. The sera from rabbits immunized with the avirulent organisms did not precipitate with either the homologous or the heterologous types of polysaccharides, and further immunization of these animals did not elicit any response.

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[1] Murray, J. F., *J. Path. Bact.,* 1935, **41**, 439.

[2] Ewing, J. O., *Ibid.,* 1933, **37**, 345.

[3] Keogh, E. V., Simmons, R. T., and Anderson, G., *Ibid.,* 1938, **46**, 565.

[4] Anderson, J. S., Happold, F. C., McLeod, J. W., and Thomson, J. G., *Ibid.,* 1931, **34**, 667.

[5] Young, C. C., *Chinese Med. J.,* Suppl. No. 1, 1936, 143.

[6] Wong, S. C., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 107.

Most sera reacted with the homologous polysaccharides diluted 1:100,000 excepting one serum which reacted with 1:250,000 (Park 8). Corresponding titers were obtained with heterologous polysaccharides.

For further study of these group reactions, absorption-tests were carried out. Equal volumes of undiluted immune serum and the optimal dilution (1:10,000) of polysaccharide were mixed and incubated at 37°C in the waterbath for 2 hours. Then the treated serum was refrigerated overnight and centrifuged rapidly for half an hour. The supernate was tested with 1:1000 and 1:5000 dilution of the various polysaccharides. Every serum (*mitis, gravis,* or intermediate) was completely absorbed by the homologous and by each heterologous polysaccharide, including the avirulent type.

*Conclusion.* The polysaccharides of *C. diphtheriæ* appear to be shared by all the types studied.

## 10225

## Sensitization of Guinea Pigs by a Modified Form of Seibert's Tuberculoprotein Derivative.

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Denaturation of tuberculoprotein is usually accompanied by a decrease in antigenic activity and in special cases, such as in the preparation of OT (old tuberculin) and of PPD (purified protein derivative), antigenicity is usually destroyed.[1] According to Seibert[2] the lack of antigenic properties of PPD is attributed to the use of heat and of trichloracetic acid employed in its preparation. It is here shown that a modified form of Seibert's PPD can sensitize guinea pigs.

*Mycobacterium tuberculosis,* H37, was grown on Wong's modification of Henley and LeDuc's synthetic medium[3] for 4 weeks. The general procedures of preparing the tuberculoprotein were essentially those used by Seibert, but 3 changes were introduced to shorten the time of preparation: (1) glycerin was not added to the tuberculin during concentration over the waterbath; (2) the concentrated tuberculoprotein was not dialyzed through a collodion membrane but was passed through a Seitz filter and precipitated directly with 50% trichloracetic acid; (3) the precipitated protein was collected and washed with 10% trichloracetic acid until free from $SO_4$, about 15 washings being necessary. The PPD obtained in this manner has physical and chemical properties similar to those described by Seibert.

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[1] Seibert, F. B., *Am. Rev. Tuberc.*, 1934, **30**, 713.

[2] Seibert, F. B., *J. Inf. Dis.*, 1932, **51**, 383; Nat. Tuberc. Assn., 1933, 165.

[3] Wong, S. C., *J. Bact.*, 1937, **33**, 451.

Cutaneous reactions of sensitive tuberculous guinea pigs to 0.005 mg were comparable to those induced by the tablet form of PPD obtained from the Mulford Laboratory. Ten to 15 mg of our PPD injected intravenously in guinea pigs weighing about 250 g produced no harmful effects. Prolonged immunization of rabbits elicited no precipitin detectable with 1:500 dilution of the antigen but complement-fixation occurred with 1:1000 dilution of antigen and serum diluted 1:5.

Twenty-four guinea pigs of various sizes were sensitized by 2 subcutaneous injections and one intraäbdominal injection of modified PPD at 5-day intervals. The total dose was 30 mg per animal. Three weeks after the last injection the animals were tested for the presence of supersensitivity by intravenous injection of varying amounts of the tuberculoprotein. The results, presented in the first 3 columns of Table I, show that 11 out of 24 sensitized animals reacted to the intravenous injection of 5 to 10 mg. Typical anaphylactic death occurred in 4; no tuberculous infection was found at necropsy. The general picture of the reactions was typical of classical anaphylaxis and it is evident that this kind of purified tuberculin can actively sensitize guinea pigs. The sensitizing property of OT in guinea pigs was observed by Reichle and Goldblatt.[4]

TABLE I.
Active Sensitization of Guinea Pigs with Modified PPD.

| No. of animals | Result of 1st test | Result of 2d test | Total No. of animals |
|---|---|---|---|
| 4 | Death, 3 min. | | |
| 2 | Severe shock, recovered after ½ to 1 hr. | Death | 2 |
| 5 | Mod. shock, recovered after 10-20 min. | " | 3 |
| | | Mod. shock | 2 |
| 13* | No response | Death | 1 |
| | | Mod. shock | 5 |
| | | Mild " | 1 |
| | | No response | 4 |

*2 died of intercurrent infection after the first test.

The animals who survived the first intravenous injection of our

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[4] Reichle, H. S., and Goldblatt, H., *Am. Rev. Tuberc.*, 1933, **27**, 291.

PPD were retested 3 weeks later with the same shocking dose (5-10 mg). At the same time cutaneous allergy, passive transfer of supersensitivity, and the effect of another shocking agent, tuberculopolysaccharide, were studied.

For the endermal test, 6 guinea pigs which had developed non-fatal shock in the previous trial and 2 normal controls were injected with 0.01 and 0.005 mg of the purified tuberculin contained in 0.1 cc of salt solution; none of the injected areas showed any evidence of reaction during a period of 24 hours.

For passive transfer of supersensitivity 3 of these animals were bled and subsequently tested for the presence of sensitization by injecting intravenously 10 mg of our PPD. Since all died of typical anaphylactic shock the sera were considered suitable for use. Accordingly, each of 3 young guinea pigs weighing about 250 g received intraäbdominally 2 cc of the pooled sera. Twenty-four hours later they were tested intravenously with 10 mg of the tuberculin. Although all of them showed a moderate degree of anaphylactic shock, recovering after 10 to 15 minutes, serological examination of the same serum revealed no precipitin or complement-fixing antibody for either the purified tuberculin or the polysaccharide prepared from the same microörganism.

Six other animals were injected intravenously with 10 mg of tuberculopolysaccharide but no symptom of shock was observed. However, when they were tested a half-hour later with 5 to 10 mg of our PPD all but one reacted. The results of the retesting of the sensitivity of the 18 animals are shown in the last column of Table I. It was found that supersensitivity returned to all the 7 animals that developed non-fatal shock in the first test. As a result of the second test 5 died of acute anaphylactic shock. Of the remaining 11 animals, 7 showed varying degrees of supersensitivity.

*Conclusion.* Although active sensitization of guinea pigs is possible under the conditions of the experiment, it is not accomplished with the same ease and regularity as with ordinary proteins. Nevertheless the antigenic nature of our PPD is evident.

## 10226 P

# Life History and Immunity Studies of the Avian Malaria Parasite, *Plasmodium circumflexum.*

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Six strains of *circumflexum* were used. These were secured from 5 different species of hosts, 3 of which were from widely separated localities (Syracuse, N. Y., Cape Cod, Mass., and Germany). The technic consisted in inoculating chronic infections (infections which had been made 2 or more months previously) of a given strain with parasites of another strain. The results indicated that a chronic infection with one strain protected against subsequent infection with another in most cases, although in general the degree of immunity to infection with another strain was somewhat less than when superinfection with the same strain was done. It was found, however, that the 4 strains originally isolated from birds caught in Syracuse (although they were isolated from 3 different host species) conferred much higher immunity against reciprocal or cross-infection with some other of the 4 than toward the strains derived from Cape Cod or German hosts. Of all 6 strains, that derived from a Cape Cod blackbird proved most able to break down the resistance conferred by chronic infection with some other strain, but previous infection with it conferred strong protection against the other five strains.

These results show that at least in this species of avian malaria immunity ("premunition") is more a matter of strain than previous work with other species had indicated (Gingrich,[1] Manwell[2]), and it was thus more like the monkey and human malaria parasites in this respect. Indeed the degree of immunity exhibited by a chronic infection of one strain towards another may be less than that occasionally observed between species. As an example, *Plasmodium rouxi* may be mentioned. This species has been found to confer a strong immunity towards subsequent infection with *circumflexum,* but the relationship is not reciprocal.

The results of the experiments to test whether immunity could be artificially conferred by the repeated injection of serum from chronic cases showed that this could be done quite successfully, thus demonstrating that avian malaria is like that in monkeys in this respect (Coggeshall and Kumm[3]). A total of 20 birds were used in these experiments, of which 14 were treated and 6 were controls. Six of the former group received 8 injections of immune serum before inoculation with parasites and one injection afterward. No infection developed in any of the 6, and this was confirmed by successful infection later. Inoculation of clean birds with the blood of these birds also gave negative results. The other 8 treated birds received 10 injections of immune sera after inoculation with parasites but in this case protection was less effective. However, all showed a definite degree of immunity as compared with the controls. It may be noted, however, that immunity was greater in those birds which received parasites of the same strain as originally used to infect the donors of the immune serum. When parasites of strain "E" (from the Cape Cod redwing referred to above) were given to birds immunized with serum from birds carrying chronic infections with one of 3 strains of Syracuse origin more severe infections developed, although still considerably milder than in the unprotected controls. It is thus clear that other factors than the reticulo-endothelial system are involved in the development of immunity.

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[1] Gingrich, W., *J. Prev. Med.,* 1932, **6**, 197.

[2] Manwell, Reginald D., *Am. J. Hyg.,* 1938, **27**, 196.

The final portion of the work herein reported concerned the possible occurrence of exoerythrocytic stages in *Plasmodium circumflexum.* Such stages have been reported for 4 other species of avian malaria *(gallinaceum, praecox, cathemerium, elongatum).* It was found that stages very similar to those said to exist in the species just listed also occur in *circumflexum,* and are most numerous in the large monocytes of the lungs, liver, spleen, and bone marrow, more or less in the order named, They may also be found in other situations, such as the heart muscle, and they may occur in large numbers in the endothelial cells of the brain. Reproduction in these forms seems always to be by schizogony, and in this respect these parasites differ from *Toxoplasma.* The latter is said to reproduce by binary fission in all the species so far worked on (although the number of these is still very small). We have confirmed this observation for the strain worked on by Sabin and Olitzky of the Rockefeller Institute, from preparations furnished through the courtesy of the former. It may also be noted that although *Toxoplasma* is a very common parasite, at least among English sparrows in this vicinity, it differs considerably from the exoerythrocytic stages seen in *Plasmodium circumflexum* infections, and also from those seen on slides of *Plasmodium cathemerium* sent by Dr. Kikuth of Elberfeld, Germany. It is only fair to admit, however, that the question of the significance of what have been regarded as exoerythrocytic stages by others, and are so regarded by the authors in the *Plasmodium circumflexum* infections mentioned here, will not be altogether conclusively settled until typical malaria infections are transferred to clean birds by inoculation of these stages alone. It is of interest that these stages have so far been observed only in acute cases, and not in all of these. Fatal cases are most likely to exhibit them.

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[3] Coggeshall, L. T., and Kumm, H. W., *J. Exp. Med.*, 1937, **66**, 177.

### 10227

## Failure of Ascorbic Acid to Influence Albuminuria and Hematuria in Nephritis.*

MAX MILLER, S. M. JOHNSTON AND J. M. HAYMAN, JR.
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Following the isolation and identification of Vitamin "C" in 1932, several reports on the effectiveness of ascorbic acid on hematuria of acute nephritis and from other causes appeared in the European literature. Szent-György[1] reported favorable results in cases of hemorrhagic nephritis following 150-200 mg of ascorbic acid on alternate days. Koranyi and Bentsath[2] also noted a hemostatic effect in nephritic hematuria. Engelkes[3] described 2 cases of hematuria and albuminuria in which the red blood cells in the urine disappeared in 10 and 6 days, respectively, after administration of 100-200 mg daily. Ceruti and Costanzo[4] stated that in 2 patients suffering from nephritis and essential forms of hematuria, 50 mg were given daily for 10 days with satisfactory results verified by microscopic studies of the urine. These favorable reports prompted Murphy[5] to advocate routine administration of ascorbic acid in cases of acute nephritis with hematuria. On the other hand, Parsons,[6] Wilbur,[7] and Abt and Farmer[8] in reviewing the subject of Vitamin

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* Supported by a grant from the Commonwealth Fund.

[1] Quoted in Wright, I. S., and Lilienfeld, A., *Arch. Int. Med.*, 1936, **57**, 241.

[2] Koranyi, A., and Bentsath, A., *Orvosi hetilap.*, 1935, **79**, 378.

[3] Engelkes, H., *Lancet*, 1935, **2**, 1285.

[4] Ceruti, G., and Costanzo, F., *Riforma Medica*, 1937, **53**, 1770.

[5] Murphy, F. D., *International Clinics*, 1938, **2**, 231.

[6] Parsons, L. G., *Lancet*, 1938, **1**, 123.

[7] Wilbur, D. L., *Arch. Int. Med.*, 1937, **59**, 512.

[8] Abt, A. F., and Farmer, C. J., *J. A. M. A.*, 1938, **111**, 1555.

"C", have stated that there is no real evidence that Vitamin "C" is of any value in any other condition than scurvy.

In order to determine definitely whether ascorbic acid did or did not have any therapeutic value in controlling hematuria in nephritis, 6 patients with acute and subacute nephritis were studied. Ascorbic acid excretion was determined by titration with 2,6-dichlorophenol-indophenol after standardization with known solutions of crystallin ascorbic acid. Hematuria was estimated by daily Addis counts on the 12-hour night urine or by hemoglobin determinations when there was gross hematuria. Dr. R. F. Hanzel kindly made the hemoglobin determinations using a modification of Bing and Baker's benzidine method. Protein was determined by Shevky and Stafford's method. In order to minimize oxidation of the ascorbic acid, urine voided during the day was collected in bottles containing glacial acetic acid. Since this would hemolyze red cells and prevent counting, the night urine was preserved with toluol. All specimens were kept in the dark in the ice box and analyzed early the next morning. After a control period of 3 to 6 days, Merck's "cebione"† was given daily for a week or 10 days. One patient received 100 mg intravenously and the others 1 gm by mouth daily.

TABLE I.
Effect of Ascorbic Acid on Hematuria and Albuminuria in Nephritis.

| | | Control Period | | | Ascorbic Acid Period | | | After Period | |
|---|---|---|---|---|---|---|---|---|---|
| Name | Age Sex | Prot. g 12 hr | RBC $10^6$ 12 hr | Ascorbic Acid mg 24 hr | Prot. g 12 hr | RBC $10^6$ 12 hr | Ascorbic Acid mg 24 hr | Prot. g 12 hr | RBC $10^6$ 12 hr |
| MB | 27F | .05 | 1.9 | 19.1 | .06 | 1.1 | 536.7 | .13 | 1.0 |
| AR* | 20F | .98 | 5.9 | 6.4 | 1.43 | 4.0 | 52.3 | 1.06 | 4.2 |
| MW | 28F | 1.95 | *500 | 22.2 | .81 | 249 | 238.2 | .45 | 106 |
| TK | 16M | 3.11 | 3.3 | 24.7 | 3.46 | 3.6 | 394.9 | 2.37 | 6.5 |
| EB | 26F | .08 | 2.9 | 14.5 | — | — | 541.5 | 1.86 | 2.4 |
| AB | 15M | 7.83 | †.34 | 12.6 | 11.77 | †1.01 | 245.1 | 10.85 | †.76 |

*100 mg intravenously.
†g hemoglobin per 12 hr.

The results are presented in summary form. Table I shows the average values obtained during the control period, during the administration of ascorbic acid and for the first 2 to 4 days after its withdrawal. In one case the daily excretion of ascorbic acid was below 10 mg, a point below the level said by Harris[9] to indicate deficiency; 3 were below 20 mg, indicating low intake. In all cases, however, administration of large doses of ascorbic acid resulted in greatly augmented excretion within the first 2 days, which would indicate that there was no marked unsaturation of the body. No hemorrhagic skin manifestations were present. In 3 cases, the amount of hematuria remained the same or increased; in 2 cases there was a slight but not significant decrease, and in one case (M.W.) there was definite improvement, although the urine did not clear up by any means. This, however, may well have been coincidental since subsidence of hematuria in acute nephritis is the rule rather than the exception. Subsequent follow-up of these 6 cases showed that 3 of the 6, including this last one, finally cleared the hematuria. The amount of albuminuria was not altered during administration of ascorbic acid, except in M.W.

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† Kindly furnished by Merck & Co.

[9] Harris, L. J., and Ray, S. N., *Lancet*, 1935, **1**, 71.

Our studies have been unable to confirm the benefits of ascorbic acid on nephritic hematuria reported in the literature. While this study was in progress, Walther[10] reported negative results similar to ours in 3 cases of hematuria and glomerulo-nephritis. It is our opinion that the administration of Vitamin "C" is not indicated for the hematuria of nephritis unless there is clear-cut evidence of Vitamin "C" deficiency.

*Summary.* In 5 patients with hematuria due to acute or subacute nephritis, the administration of ascorbic acid in massive doses over a period of 6 to 10 days sufficient to saturate the body stores had no significant effect on the amount of hematuria or albuminuria. In one case there was a definite decrease but coincidental spontaneous improvement could not be excluded.

### 10228

## Diluted and Undiluted Diphtheria Toxoid as Immunizing Agents in Man.

Hortense B. Schmitz. (Introduced by James A. Doull.)

*From the Department of Hygiene and Bacteriology, School of Medicine, Western Reserve University.*

Hartley[1] observed that guinea pigs injected with diluted toxoid attained a higher immunity to diphtheria toxin than those receiving an equivalent amount of undiluted toxoid. These results were confirmed by Timmerman and Brandwijk.[2] The present study was undertaken to discover the relationship of concentration of toxoid to antitoxin-production in human subjects.

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[10] Walther, G., *Med. Klin.* 1938, **8**, 260.

[1] Hartley, P., *Brit. J. Exp. Path.*, 1935, **16**, 468.

[2] Timmerman, W. A., and Brandwijk, A. C., *Brit. J. Exp. Path.*, 1936, **17**, 252.

Thirty-three medical students were selected in the spring of 1936, each of whom had less than 0.002 units of antitoxin per cc of blood serum. In the fall, each was tested for sensitivity to toxoid by the Moloney[3] test; 7 reacted positively and were eliminated. The names of the remaining 26 students were listed alphabetically and divided alternately into 2 groups; Group A students to receive diluted, and Group B, undiluted toxoid. The first injection was given on November 3, 1936, three weeks after the test for sensitivity. Each student was bled at this time. Two in Group A and one in Group B were found to show a rise in antitoxin on this bleeding, perhaps due to the stimulus of the Moloney test. These were eliminated, leaving 11 in Group A and 12 in Group B. Thus there were excluded from the experiment all individuals having more than 0.002 units per cc and all responding to the Moloney test either by cutaneous reaction or production of antitoxin. One member of Group A did not finish the experiment, leaving 10 in this group.

The members of Group A received 3 injections of 0.5 cc each of toxoid which had been diluted to 5 cc with saline. Those in Group B were injected with 0.5 cc of undiluted toxoid. Injections were given subcutaneously at 6-week intervals. The toxoid was supplied by Dr. D. T. Fraser of the Connaught Laboratories, University of Toronto, and contained 14 $L_f$ per cc. Subsequently, specimens of blood were obtained on 10 occasions over a period of about 1½ years; every 2 weeks for the first 6 weeks; every 3 weeks for the next 6 weeks; once a month for the next 3 months, and twice more at longer intervals. No Schick or Moloney tests were given during this period.

Antitoxin was titrated according to Fraser's[4] modification of the original Römer method. The sera were tested for 0.002, 0.004, 0.01, 0.02, 0.05, 0.1, 0.5, 1, 2, and 5 units of antitoxin.

Table I is sufficient to illustrate the results. All individuals showed a rise in antitoxin-level. In Group A, 3 showed increased titers after the first injection and 7 others after the second. In Group B, 4 showed increased titers after the first injection and 8 after the second.

Among those responding to the first injection, the intervals to first response were similar in both groups. In Group A one individual increased his titer within 2 weeks, one between the second and

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[3] Moloney, P. J., and Fraser, C. J., *Am. J. Pub. Health*, 1927, **17**, 1027.

[4] Fraser, D. T., *Trans. Roy. Soc. Can.*, 1931, Sect. V, p. 175.

TABLE I.

Distribution of Medical Students According to Diphtheria Antitoxin Titers at Various Periods Following Toxoid-injections. All Titers at Time of First Injection Were Less than .002. Group A: Diluted toxoid 1:10. Group B: Undiluted toxoid.

| Antitoxin level (units per cc) | 4 wk after *first* injection | | 3 wk after *second* injection | | 4 wk after *third* injection | | 68 wk after *third* injection | |
|---|---|---|---|---|---|---|---|---|
| | A | B* | A | B | A | B | A | B |
| Less than 0.002 | 8 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.002-0.01 | 1 | 0 | 2 | 3 | 0 | 0 | 2 | 3 |
| 0.01-0.1 | 1 | 1 | 4 | 1 | 1 | 2 | 5 | 4 |
| 0.1-1.0 | 0 | 1 | 3 | 4 | 6 | 5 | 3 | 5 |
| More than 1.0 | 0 | 0 | 1 | 4 | 3 | 5 | 0 | 0 |

*One serum not titrated.

fourth, and one between the fourth and sixth. All save 2 of those responding to the first dose of toxoid ultimately showed relatively higher levels of antitoxin, that is, over 2 units.

All 15 individuals who did not respond until after the second injection showed rises in titer within 3 weeks after the second dose of toxoid. After the third dose, all but 3 showed further increase. The highest levels reached by these 15 were on the average substantially lower than for those who responded to the first dose of toxoid, but there was no significant difference between the groups.

On the final titration, all members of both groups had fallen from the highest point, but retained significant amounts of antitoxin.

Two individuals who had been previously immunized with toxin-antitoxin were among those responding to the first injection of toxoid. Two others who had had diphtheria responded to the second dose and showed no significant variations in immune response from others in their groups.

Taken by and large, no differences were observed either in rapidity of response or in amount of antitoxin produced between a group of young adults treated with undiluted diphtheria toxoid and a comparable group given the same toxoid diluted 10 times with normal saline. Furthermore, the rate of loss of antitoxin from the blood stream was about the same in both groups although individuals varied greatly in this respect.

### 10229 P

## Revival of Frog's Spermatozoa Vitrified in Liquid Air.

Basile J. Luyet and Eugene L. Hodapp.
*From the Biology Department, St. Louis University.*

It is now known that water in aqueous colloids takes the crystalline state at temperatures extending from 0°C to some tens of degrees below zero and that, when exposed, while still liquid, to lower temperatures, it takes the vitreous state.[1] Using the method of immersion in liquid air for vitrification it was found that a colloid which contains 90% water when spread on a microscope coverslip 1/10 mm thick, can be vitrified if the thickness of the layer treated does not exceed a few micra, while when the water content decreases to 50%, the vitrifiable thickness increases to 0.2 mm.[2] The assumption that vitrification does not disintegrate living matter as crystallization does was previously verified in some plant cells.[3] In this paper it will be shown that in animal cells, in which the motility can be taken as a sign of vitality, life is not destroyed by the vitrification treatment.

In a first series of experiments, excised frog testes were cut open and the spermatozoa expressed through the cut end were smeared on an ordinary coverslip. This was then immersed in liquid air for about 10 seconds and immediately after in pond water at +20°C in order to insure a rapid warming and in that manner avoid crystallization during the warming through the dangerous zone of temperatures. The spermatozoa left on the slide or dispersed in the pond water were all dead.

One might have suspected that the 20 degree interval (from 0° to +20°) allowed for rapid warming did not constitute a large enough temperature difference, but since our experiments with plant epidermis[3] and with moss leaves[4] were successful with a warming bath at +20° we suspected the high heat capacity of the too thick coverslips to be responsible for a too slow cooling or warming. Mica sheets split to a thickness of about 10 micra were then substituted for the glass plates, but still no spermatozoon could be revived.

A reduction of the water content by plasmolysis, which proved successful in plant material[3, 4] was then attempted. Solutions containing the Ringer's constituents at higher concentrations were found injurious before they became efficient. But 1 M sucrose was well supported by the spermatozoa and when the organisms, bathed in that solution for 3 minutes, and mounted on a mica film, were immersed in liquid air for 10 seconds and immediately afterwards dipped in pond water at +20° some 20% of them resumed motion (30 experiments). When a 2 M concentration of sucrose was used, about 40% of the spermatozoa ceased moving during the plasmolysing time of 3 minutes, under the injurious action of sucrose alone; but about 100% of those which survived exposure to sucrose, survived vitrification in liquid air (50 experiments). At concentrations between 1 M and 2 M, intermediate results were obtained (8 experiments).

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[1] Luyet, B., *Biodyn.*, 1937, **29**, 1.

[2] *Loc. cit.*, p. 7.

[3] Luyet, B. J., and Thoennes, G., *Science*, 1938, **88**, 284.

[4] Luyet, B., and Gehenio, P. M., in press.

If, instead of immersing the preparation in water for rapid warming, we let it warm up slowly in the air all the spermatozoa were dead no matter what sugar concentration was used to plasmolyse them Frozen spermatozoa do not only stop moving, they show signs of disintegration, losing their refringence and appearing broader.

The time of immersion in liquid air was varied from 2 seconds to one hour without any difference in the results.

The revived spermatozoa, transferred in the reviving process from sugar solution into pond water, maintained their motility as long as the controls (plasmolysed and deplasmolysed but not exposed to liquid air). In one case the motility was observed 12 hours after exposure.

The use of a liquid non-miscible with water, for example, isopentane, as a warming fluid after vitrification presented the advantage of avoiding the dispersion of the organisms from their mica support but a serious inconvenience was experienced in the use of such liquids either as cooling or as warming media, namely, that they are considerably slower in their effect than water, probably on account of their lower heat conductivity and, in particular, on account of the low "contact conductivity" at the interface where they do not mix with water.

## 10230

# Chemical Concentration of Mammogen from Prehypophyseal Tissue.*

A. A. Lewis and C. W. Turner.

*From the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Columbia.*

With the development of an assay technic for the duct-growth entity of mammogen,[1] our attention has turned to the study of methods of extraction and purification. In previous reports from this laboratory,[2,3] a method has been described for the extraction of the lactogenic and carbohydrate-metabolism hormones. In order to determine whether this method would be equally satisfactory for the extraction of mammogen, the following procedure was carried out.

Pituitary tissue from pregnant cattle was dried with acetone and ether. Assay showed there had occurred a loss of 60% of mammogen. The dried tissue was then extracted with 60% alcohol at a pH of 9 to 10 and the *initial extract* precipitated by adjusting the pH to 5.7 and increasing the alcohol content to 86%. As the *initial extract* contained only 5% as much activity as the fresh tissue, it was clear that this method was unsatisfactory. Further, the residual tissue was found to contain slightly more mammogen per unit than did the initial extract.

In a second experiment, the fresh pituitary tissue was not dried, but immediately extracted with alcohol and the *initial extract* precipitated as described above. The 86% alcohol solution was then vacuum distilled. The oily residue contained a mouse unit per 3 mg. It was estimated that about 90% of the mammogen present in the fresh tissue was recovered.

As the mammogenic hormone appeared to be soluble in the lipid solvents, several methods were tried. Extraction with 95% alcohol for 2 days, followed by vacuum distillation produced a residue containing slightly less than 1 mouse unit in 40 mg. Extraction with several volumes of hot ether-alcohol (1:3), resulted in a preparation containing 1 unit per 3 to 4 mg which was equivalent to about 100% of the potency of the fresh tissue.

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* Journal series No. 581. Aided in part by a grant from the International Cancer Research Foundation.

[1] Lewis, A. A., Turner, C. W., and Gomez, E. T., *Endocrinology*, 1938, **23**, 672.

[2] Bergman, A. J., and Turner, C. W., *J. Biol. Chem.*, 1937, **118**, 247.

[3] Bergman, A. J., and Turner, C. W., *J. Biol. Chem.*, 1938, **123**, 471.

TABLE I.
Potency of Mammogen in Extracts of Prehypophyseal Tissue from Pregnant Cattle.

| Extract | No. of mice | Days injected | Total dose, mg | No. of mice showing | |
|---|---|---|---|---|---|
| | | | | mammary stimulation | no mammary stimulation |
| Fresh prehypophysis from pregnant cattle | 53 | 6 | 105 | 30 | 27 |
| Acetone and ether dried prehypophysis | 8 | 14 | 28 | 0 | 8 |
| | 12 | 6 | 40 | 5 | 7 |
| Initial extract | 4 | 21 | 21 | 0 | 4 |
| | 12 | 6 | 120 | 6 | 6 |
| Inert material (for most pituitary hormones) | 3 | 4 | 40 | 0 | 3 |
| | 3 | 4 | 80 | 1 | 2 |
| | 10 | 6 | 96 | 5 | 5 |
| Residue—86% alcohol distillation; second extraction | 12 | 6 | 1 | 3 | 9 |
| | 11 | 6 | 3 | 5 | 6 |
| Residue—95% alcohol distillation; cold extraction | 5 | 6 | 20 | 1 | 4 |
| | 8 | 6 | 40 | 3 | 5 |
| Residue—95% alcohol distillation and ether-hot extraction | 10 | 6 | 3 | 3 1? | 6 |
| | 11 | 6 | 4 | 7 | 4 |

*Summary.* Acetone and ether drying of prehypophyseal tissue from pregnant cattle was observed to result in a 60% loss of mammogenic hormone. Similarly mammogen was not precipitated to any extent from 86% alcohol at pH 5.7. The residues obtained from the vacuum distillation of 86% alcohol and from hot alcohol-ether extraction were potent in mammogen.

These observations indicate that mammogen has distinctly different chemical properties from the lactogenic and carbohydrate-metabolism hormones which are isoelectrically precipitated in alcohol. Mammogen appears to be extracted in lipid solvents and remains in solution at high concentrations of alcohol. It can be recovered as an oily residue upon the vacuum distillation of the alcohol or ether-alcohol extracting solutions.

## 10231 P

## Asphyxia of the Spinal Cord of the Cat.

A. VAN HARREVELD AND GEORGE MARMONT.

*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena.*

The effect of the onset of asphyxia of the spinal cord has been investigated frequently. Usually a period of increased reflex excitability during the development of asphyxia has been described. In a series of experiments we studied the recovery of reflex activity after various periods of asphyxia. The cord was asphyxiated by bringing it under a pressure higher than that of the blood, thus excluding the blood supply. The spinal cord was ligated in the lower thoracic region 5 days previous to the planned experiment, without opening the dura. A cannula was brought in the subdural cavity of the lower part of the cord, which was separated from the rest of the central nervous system by the ligature. Deoxygenated physiological solution was then forced into the cavity under a pressure of 23-25 cm of mercury. The cord was kept under pressure for periods varying between 25 and 75 minutes. The animals were observed up to 3 weeks afterwards.

After 75 minutes of asphyxia no reflex activity returned; after 25 minutes of asphyxia all the reflexes of the hind limb and the tail returned within 2 hours, and these reflexes resembled those in the normal animal except that their excitability was increased. As the time of asphyxia was lengthened in 10 minute increments more and more reflexes did not return. First the skin reflexes were lost, then the tendon reflexes, and finally tone. The excitability of the tendon reflexes when their return was observed was usually much higher than normal. The tone in the extensor muscles of the legs was greatly enhanced in many cases, even in some instances to such a degree that it resembled rigor mortis. This tone could continue for days or even weeks; it was proven to be reflex in origin because transection of the dorsal roots of the lumbo-sacral cord caused its disappearance. After the longer periods of asphyxia (55 and 65 minutes) the return of tone and tendon reflexes was only temporary, disappearing again after 48 hours.

Histological preparations, made 3 weeks later, of the cord of animals subjected to pressure for the longer times revealed the presence of only about 5% of the normal number of perikarya. Therefore it seems probable that the temporary reappearance of reflex activity must be ascribed to the temporary recovery of conduction in cells which are damaged so severely that they will die shortly.

Since the high tone could continue for weeks following asphyxia, it is concluded that it is a release phenomenon. By this it is meant that a system normally inhibiting the tone is damaged to a greater extent than the excitatory component of the tone reflex. Normal tone would be an equilibrium between an excitatory and an inhibitory component of this reflex. The increased excitability of the tendon reflexes is to be explained in a similar manner.

Since the inhibitory systems seem to be less resistant against asphyxia and would therefore be abolished first during the development of asphyxia, the increased reflex excitability which has been described by various authors must be considered a release phenomenon also.

### 10232

## Effects of Nicotinic Acid on Specific Antibody Production.

R. R. MADISON,* MARGARET FISH AND OLIVER FRICK. (Introduced by W. H. Manwaring.)

*From the Laboratory of Bacteriology and Experimental Pathology, Stanford University, California.*

In a previous paper[1] it was shown that ascorbic acid mixed with or injected simultaneously with horse serum hastens and augments specific-precipitin production in rabbits. We have extended this work to include other "activators" of tissue-enzymes, the present paper summarizing the "co-antigenic" effects of sodium salts of nicotinic acid.

In our initial tests with this activator six 2000 g rabbits were injected intravenously with 0.5 cc horse serum. Three of these animals were then given intravenously a fresh mixture of 50 mg nicotinic acid plus 22 mg $Na_2CO_3$ in 1 cc NaCl solution. The rabbits were bled at frequent intervals, and the average precipitin-titer (ring test) was determined for each group. Data thus obtained are recorded in Table I.

The results in this preliminary series are similar to those pre-

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* Parke, Davis and Co. Research Associate in Immunology, Stanford University, California.

[1] Madison, R. R., and Manwaring, W. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 402.

TABLE I.
Co-antigenic Action of Nicotinic Acid.

| Avg titer per group | 7th day | 11th day | 14th day | 18th day | 26th day |
|---|---|---|---|---|---|
| Nicotinic-acid group | 1:1850 | 1:5500 | 1:7700 | 1:8300 | 1:10000 |
| Control group | 1:700 | 1:2100 | 1:1800 | 1:2300 | 1:2700 |

viously obtained with Vitamin C, except that the percentile increase in antibody-production is less than with ascorbic acid.

In order to determine the optimal antibody-stimulating dose of nicotinic acid, 21 rabbits were divided into 7 groups of 3 each. Each animal was given a single intravenous injection of 0.5 cc horse serum, mixed with varying amounts of freshly neutralized nicotinic acid. The average titers for 4 of the 7 groups are recorded in Fig. 1.

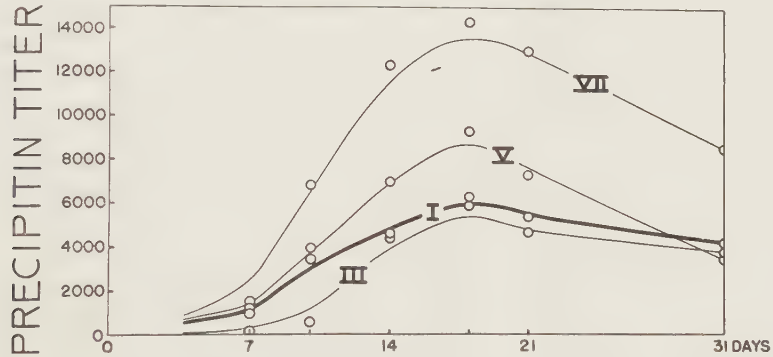

FIG. 1.

Effects of Varying Amounts of Nicotinic Acid on Antibody-production.
Group I—Control group, no nicotinic acid.
Group III—25 mg nicotinic acid.
Group V—100 mg nicotinic acid.
Group VII—800 mg nicotinic acid.
Intermediate groups II (12.5 mg), IV (50 mg), and VI (300 mg) gave data intermediate between those recorded in the figure.

Fig. 1 shows that under the conditions of the test, relatively small doses of sodium nicotinate inhibit specific-precipitin production. Medium doses may be without demonstrable effect, while massive doses augment antibody-production. Even with massive doses, however, antibody stimulation is less pronounced than that previously reported with ascorbic acid.

No theory is suggested to account for this dual effect. The phenomenon, however, is reminiscent of dual effects reported by Walbum, *et al.*, in their studies of the antibody-stimulating or inhibiting effects of metallic salts.

Since such massive doses of nicotinic acid are not clinically feasible, study of nicotinic acid as a possible adjuvant in vaccine-therapy was discontinued at this point.

## 10233

# Biphasic Effect of Male Sex Hormone on the Pituitary of the Female Rat.

S. C. Freed, J. P. Greenhill and S. Soskin.

*From the Department of Metabolism and Endocrinology,* Michael Reese Hospital, Chicago, Illinois.*

It is known that testosterone and testosterone propionate are capable of suppressing the cyclic activity of the ovaries in several species of animals.[1-4] Because rats which were treated with these substances presented ovaries with large corpora lutea, it was suggested that the androgenic hormones caused a release of the luteinizing principle from the anterior pituitary. This, in turn, gave rise to the persistent corpora lutea as in the diestrus of pregnancy or lactation. In monkeys and humans, on the other hand, similar treatment resulted in a cessation of ovulation and some degree of ovarian atrophy. This apparent species difference in the response to testosterone was also reflected in the uterus. The uteri of rats and rabbits developed progestation-like changes,[5, 6] while the uteri of the primates underwent involution.[3, 7]

It seemed to us to be significant that Hertz and Meyer,[8] who used 1/10 to 1/20 of the amounts of testosterone propionate which had been employed by others to induce persistent corpora lutea, showed that these small doses effectively inhibited the secretion of the follicle-stimulating hormone in parabiotic rats. This suggested that a qualitative difference in pituitary response to large and small doses of androgen might account for the confusing variation in the results which had been obtained. The increasing clinical use of androgenic therapy for certain ovarian dysfunctions lent practical as well as theoretical importance to further investigation.

Groups of adult female rats, showing regular estrous cycles, were given daily subcutaneous injections of testosterone propionate for

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* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Robson, J. M., Proc. Soc. Exp. Biol. and Med., 1936, **35**, 49.

[2] Browman, L. G., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 205.

[3] Zuckerman, S., *Lancet*, 1937, **2**, 676.

[4] Papanicolaou, G. V., Proc. Soc. Exp. Biol. and Med., 1938, **37**, 689.

[5] Klein, M., and Parker, H. S., *Proc. Roy. Soc. Lond. s. B.*, 1937, **121**, 574.

[6] Brooksby, J. B., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 235.

[7] Gaines, J. A., Salmon, U. J., and Geist, S. H., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 779.

[8] Hertz, R., and Meyer, R. K., *Endocrinology*, 1937, **21**, 756.

16 days. A different daily dose was used for each group, and vaginal smears were made on each animal every day. In the groups of rats receiving 1.0 mg and 0.1 mg per day vaginal cornification was completely suppressed throughout the injection period. The group which received 0.05 mg daily, showed only an occasional estrus smear. All the animals were autopsied on the 17th day. The ovaries and uteri were weighed, and examined both grossly and microscopically.

Table I shows the marked difference in ovarian and uterine weights which resulted from the different dosages employed. Except for the slight difference in vaginal cornification mentioned above, the results obtained with 0.1 mg per day resembled those obtained with the smallest dosage and need not be described separately. It may be seen that as little as 0.05 mg of testosterone propionate per day caused severe ovarian atrophy, and uteri significantly below normal in weight. The ovaries of the rats treated with 1.0 mg doses were much larger, though still somewhat below normal weight. Their uteri showed pronounced growth, as previously reported by others.

TABLE I.
Effect of Administration of Testosterone Propionate for 16 Days in the Adult Rat.

| Daily dose, mg | No. of rats | Avg wt of ovaries, mg | Avg wt of uterus, mg |
|---|---|---|---|
| 1.0 | 7 | 56 | 570 |
| 0.1 | 8 | 32 | 298 |
| .05 | 8 | .24 | 262 |
| Control | 6 | 74 | 370 |

Gross and microscopic examination of the ovaries and uteri showed just as marked differences in the effects of small and large doses of the androgen. The small ovaries of the rats treated with 0.05 mg doses, failed to show any normal structures on gross examination. Under the microscope, a few small follicles were seen, while corpora lutea were entirely absent. The uteri of these animals were much smaller in diameter than the normal diestrus uterus, though not quite as small as the castrate uterus. Microscopically, the uterine endometrium was atrophic, with low cuboidal epithelium and poorly developed glands, although there were some slight decidua-like changes in the stroma. In contrast to the foregoing, the ovaries of the rats which had received the 1.0 mg dosage contained numerous large corpora lutea, which were evident grossly as well as microscopically. The large uteri of these animals exhibited an endometrium with progestation-like appearance. The glands were well developed and actively secretory. There were decidua-like cells in the stroma.

Our results show that both the effects previously found in rodents and those reported in primates, can be obtained in the same species (rats) by varying the dosage of testosterone propionate. This explains the apparently contradictory results which have been obtained with this hormone, and obviates the necessity for postulating a species difference in its action. The estrus-suppressing effects in our rats were obtained with much smaller doses of testosterone propionate than have hitherto been used. It seems likely that the similar results which others have obtained in primates, were due to the arbitrary choice of a relatively small dosage for those experiments.

The different effects of the large and small doses of the androgen may be explained by the different actions of these doses on the anterior hypophysis, and by the direct effects of the amount of androgen injected. Both large and small doses depress the elaboration of follicle-stimulating hormone by the pituitary, as judged by the cessation of estrous cycles. Large doses, however, also cause a release of luteinizing hormone, as indicated by the numerous large corpora lutea in the ovaries. This difference in the effects of large and small doses on the pituitary is similar to that which is known to occur with the estrogens.[9] Thus a small dose of testosterone results in cessation of the estrous cycles, ovarian atrophy, diminished estrin and involution of the uterus. A large dose results in cessation of the estrous cycles, persistent corpora lutea and diminished estrin. But uterine involution does not occur because the larger amount of androgen present acts directly on the uterus to prevent the atrophy and to produce the progestation-like changes.[5, 6]

## 10234

## Utilization of Carbohydrate by the Phlorhizinized Dog.*

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The glycosuria produced by the administration of phlorhizin has served as an experimental tool in the study of carbohydrate metabo-

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[9] Hohling, W., *Klin. Wchnschr.*, 1934, **13**, 97.

* Aided by the Max Pam Fund for Metabolic Research.

lism. The scientific literature on the subject, however, presents an anomalous situation as regards the ability of the phlorhizinized animal to utilize sugar. On the basis of characteristic R.Q. values and urinary D:N ratios, Lusk and others[1] concluded that so-called phlorhizin diabetes is essentially similar to pancreatic diabetes, in that no carbohydrate is utilized by either type of animal. However, later work has shown that the phlorhizinized dog exhibits every conventionally accepted criterion of considerable ability to utilize carbohydrate. Thus administration of glucose raises the R.Q., diminishes ketosis,[2, 3] spares protein breakdown,[4] and leads to a synthesis of glycogen.[3] Isolated tissues from phlorhizinized animals, react to a glucose medium with a rise in R.Q. similar to that exhibited by tissues from normal animals.[5]

A parallel situation has existed in regard to the question of the ability of depancreatized animals to utilize sugar. Here too, the characteristic R.Q. of 0.7 and the D:N ratio of about 2.8 were put forward as proof that the depancreatized dog does not utilize carbohydrate. But, as in the case of phlorhizin diabetes, evidence is accumulating which shows that the depancreatized animal is well able to make use of sugar. Removal of the liver from a depancreatized dog leads to a similar fall in the blood sugar level as occurs after hepatectomy in the normal animal. Glucose has to be given to prevent hypoglycemia and maintain life.[6] It has been shown that completely depancreatized dogs maintained without insulin, react to glucose administration with a rise in R.Q., a diminution of ketosis and a sparing of protein.[7, 8] More recently we have shown that carbohydrate utilization is directly related to the height of the blood sugar level, in the depancreatized as well as in the normal dog. At his usual hyperglycemic blood sugar level the diabetic dog uses as much or even more carbohydrate than does the normal dog at his normal blood level.[9]

It is obvious that phlorhizination and pancreatectomy produce metabolic disturbances which are in many ways similar to each other, but which differ in one important respect: pancreatectomy is followed by hyperglycemia, phlorhizin administration by hypoglycemia. In view of our findings that carbohydrate utilization is directly related to the height of the blood sugar level, it seemed important to study the use of sugar by the phlorhizinized animal, taking this relationship into account.

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[1] Lusk, G., *The Science of Nutrition,* F. Lippincott Co., Philadelphia, 1925.

[2] (a) Wierzuchowski, M., *J. Biol. Chem.,* 1926, **68**, 385; (b) Deuel, H. J., Wilson, H. E. C., and Milhorat, A. T., *J. Biol. Chem.,* 1927, **74**, 265.

[3] Nash, T. P., *Physiol. Rev.,* 1927, **7**, 385.

[4] Mirsky, I. A., Heiman, J. D., and Swadesh, S., *Am. J. Physiol.,* 1937, **120**, 681.

[5] Shorr, E., Loebel, R. O., and Richardson, H. B., *J. Biol. Chem.,* 1930, **86**, 529.

[6] Mann, F. C., and Magath, T. B., *Arch. Int. Med.,* 1923, **31**, 797.

[7] Soskin, S., *J. Nutrition,* 1930, **3**, 99.

[8] Ring, G. C., *Am. J. Physiol.,* 1934, **109**, 88.

[9] Soskin, S., and Levine, R., *Am. J. Physiol.,* 1937, **120**, 761.

Dogs were fasted for 3 days and eviscerated on the morning of the fourth day. During this period, 4 daily doses of 1 g of phlorhizin in oil were administered; the last dose being given on the morning of the operation. The effectiveness of the phlorhizination was checked by determining the blood sugar level, and in some animals by calculating their D:N ratios. The details of sugar administration and calculation of utilization will be found in our previous publication.[9]

There were 13 successful experiments on 13 dogs (Fig. 1). All except 2 of the 13 dogs showed a rate of utilization similar to that of normal animals at the same blood sugar levels. The 2 exceptions which showed a smaller rate of utilization, also exhibited a glycogen breakdown far below the average found in the normal, depancreatized and phlorhizinized animals of the present and previous[10] series. Although we have included the results from these 2 animals in Fig. 1, it seems justifiable to exclude them from our discussion for the time being.

Table I compares the average rates of glycogen breakdown and lactic acid increase in normal, depancreatized and phlorhizinized dogs. If we take into account the lower initial glycogen values of the phlorhizinized animals, it can be seen that their rate of muscle glycogen breakdown compares to that of normal rather than to that of depancreatized animals. The rate of lactic acid accumulation shows similar relationships.

It is evident from our results that as compared with normal dogs, there is essentially no diminution in the utilization of carbohydrates by phlorhizinized animals, at blood sugar levels ranging from 9 to 800 mg %. This agrees with other evidence to the same effect, quoted in our introductory remarks. Drury[11] in a recent study of eviscerated phlorhizinized dogs, concluded that they use only one-third the normal amount of carbohydrate. However, he did not properly take into account the carbohydrate supplied by glycogen breakdown, and only measured the disappearance of sugar from the blood stream.

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[10] Soskin, S., Levine, R., and Heller, R. E., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 6.

[11] Drury, D. R., Bergman, H. C., and Greely, P. O., *Am. J. Physiol.*, 1936, **117**, 323.

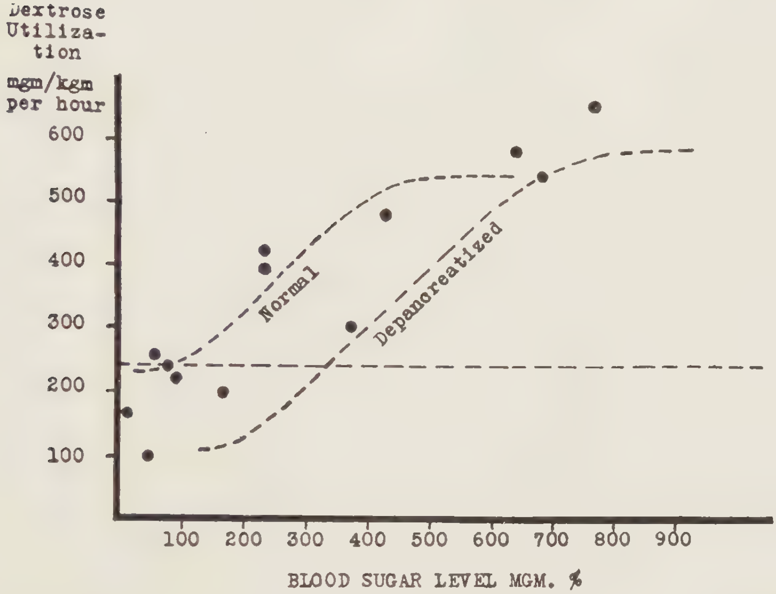

FIG. 1.

Each black dot represents an experimental result on a different phlorhizinized dog. The smooth curves for the normal and depancreatized animals, which are included for comparison, are derived from previously reported experiments.[9] The horizontal line indicates the rate of dextrose utilization of normal dogs within the normal glycemic range.

TABLE I.

| | Avg muscle glycogen | | Avg blood lactic acid | | | |
|---|---|---|---|---|---|---|
| Condition | Initial mg% | Final mg% | Initial mg% | Final mg% | Avg decrease in glycogen mg/%/hr | Avg rise in lactic acid mg/%/hr |
| Normal | 511 | 355 | 50.5 | 106.7 | 43.1 | 15.2 |
| Depancreatized | 337 | 217 | 118.1 | 183.2 | 38.4 | 21.0 |
| Phlorhizinized | 431 | 314 | 99.4 | 152.2 | 32.5 | 14.5 |

Completely phlorhizinized dogs excrete large quantities of sugar in the urine. At the same time they use normal amounts of carbohydrate. There must, therefore, be an overproduction of sugar in these animals. Hence, the low R.Q. values in these animals must be related to the overproduction rather than to an underutilization of sugar. These low R.Q.'s may result from a preponderance of reactions in which oxygen is used up without a corresponding production of carbon dioxide, such as the formation of sugar from proteins and fatty acids. That the liver of the phlorhizinized animal contributes a very low R.Q. which reduces the composite R.Q. of the whole animal, has been shown by direct observation of the perfused liver from phlorhizinized animals.[12]

In view of the fact that the utilization of carbohydrate is not inhibited by phlorhizin, the amount of sugar in the urine cannot represent all the sugar formed by the liver. It is, therefore, impossible to consider the D:N ratio of the urine as an index of the amount of sugar formed from protein or other precursors. Direct determinations on perfused livers of normal as well as phlorhizinized animals show that the D:N ratios range between 5.0 and 14.0.[12, 13] These figures present a truer picture of the derivation of sugar than the urinary D:N ratios, which are lowered by the utilization of a considerable portion of the carbohydrate formed.

We have concluded previously that the glycosuria and hyperglycemia of pancreatic diabetes are also due to an overproduction of sugar from non-carbohydrate sources. However, the causative mechanisms of overproduction are not the same in the phlorhizinized as in the depancreatized animal. The removal of the pancreas disturbs the normal regulation of hepatic glycogenesis and glycogenolysis which is dependent upon the height of the blood sugar and the presence of insulin.[14] This disturbance leads to uninhibited glycogenolysis and excessive gluconeogenesis. However, in the phlorhizinized dog, insulin is not lacking.[15] The increased hepatic glycogenolysis and gluconeogenesis are due to the persistently low blood sugar level resulting from the lowered renal threshold for sugar. This lowered threshold which is responsible for the continual glycosuria is due to the inhibition of the mechanism for the reabsorption of glucose in the kidney tubules. Recently Lundsgaard and others have shown that the transfer of sugar from the kidney tubules back to the blood stream probably occurs by way of the formation of hexose-phosphate. This phosphorylation of glucose is inhibited by phlorhizin.[16] Although all tissues are subject to this same action of phlorhizin, the kidney shows the greatest effects because it has a limited ability to destroy phlorhizin and also because the drug accumulates in the kidney in a larger concentration. Muscle tissue destroys phlorhizin very quickly and thus the phosphorylation mechanisms in muscle are not disturbed to any great extent.[17] Our results tend to confirm that this is the general rule since, except for the 2 instances mentioned above, there was no inhibition of glycogen breakdown in our animals.

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[12] Blixenkrone-Møller, N., *Ztschr. f. physiol. Chemie,* 1938, **252**, 117.

[13] Heller, H., *Acta Med. Scand.,* 1936, **90**, 489.

[14] Soskin, S., Mirsky, I. A., Zimmerman, L. M., and Heller, R. E., *Am. J. Physiol.,* 1936, **114**, 648.

[15] Nash, T. P., and Benedict, S. R., *J. Biol. Chem.,* 1924, **61**, 423.

[16] (a) Lundsgaard, E., *Biochem. Ztschr.,* 1933, **264**, 209; (b) Kalckar, H., *Enzymologia,* 1937, **2**, 47.

[17] Lambrechts, A., *Compt. Rend. Soc. Biol.,* 1935, **118**, 1248.

The excessive gluconeogenesis in both phlorhizin and pancreatic diabetes, although caused by different mechanisms, nevertheless, leads to the same consequences, namely, continued glycosuria, low liver glycogen values, the accumulation of fat in the liver, ketosis and lowered R.Q. To this extent the two conditions are similar. The presence of insulin in the phlorhizinized animal, however, enables it to maintain the normal relationship between the height of the blood sugar level and the rate of entry of blood sugar into the tissues.[9, 18] This differs from the depancreatized animal, which requires a higher blood sugar level to allow a normal rate of entry.

*Summary.* At similar blood sugar levels, the phlorhizinized dog utilizes dextrose at the same rates as does the normal animal. The similarities and differences between phlorhizin diabetes and pancreatic diabetes are briefly discussed.

## 10235 P

## Effect of Vitamin A Deficiency on the Rate of Apposition of Dentin.*

I. SCHOUR, M. C. SMITH AND M. M. HOFFMAN.

*From the Department of Histology, University of Illinois, College of Dentistry, and the Department of Nutrition, College of Agriculture, University of Arizona.*

Sixty albino rats placed on complete Vitamin-A-deficiency diet were given 2 intraperitoneal injections of 0.5 cc of a 2% solution of alizarine Red S (Table I). Alizarine is deposited as a red line in the dentin growing and calcifying at the time of each injection.[1] By measuring, in ground sections of incisors, the distance between the 2 injection effects and dividing this by the time interval daily rates of apposition were obtained.

The daily rate of normal dentin apposition averages 16 $\mu$ in both the labial and lingual portions of the tooth. In the experimental animals the rate of dentin apposition increased on the labial and

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[18] Soskin, S., and Levine, R., *Am. J. Physiol.*, 1938, **123**, 192.

* Aided by a grant from Mead Johnson and Co.

[1] Schour, I., and Hoffman, M. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 710.

TABLE I.

History of 60 Albino Rats on a Vitamin-A-Deficient Diet and the Daily Rate of Dentin Apposition in Their Incisor Teeth.

| Group No. | No. of animals in group* | Age (days) when Alizarine Red S was injected | Replacement therapy and ages (days) at which started | Age in days at death | Avg rate of apposition per 24 hr in microns | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Labial | Disto-lingual | Mesio-lingual | Mid-lingual |
| 1 | 45 | 30, 40 | None | 45 | 16.14 | 14.38 | 13.76 | 13.42 |
| | | 43, 54 | | 73 | 16.79 | 13.11 | 12.48 | 10.86 |
| | | 47, 57 | | 77 | 17.91 | 12.85 | 12.13 | 9.57 |
| | | 50, 60 | | 65 | 18.68 | 11.14 | 10.75 | 7.84 |
| | | 64, 72 | | 77 | 19.63 | 11.29 | 9.01 | 6.43 |
| 2 | 8 | 47, 51 | Suboptimum vitamin A replacement as blue gramma grass at 47 days | 52 | 18.32 | 12.47 | 11.83 | 8.91 |
| 3 | 7 | 50, 60 | Full replacement as cod-liver-oil at 50 days | 75 | 16.12 | 15.78 | 15.76 | 15.81 |
| | | 60, 70 | | 75 | 15.97 | 15.83 | 15.80 | 15.91 |

*All rats were placed on vitamin-A-deficient diet at 21 days of age (weaning).

decelerated on the lingual portions in direct proportion to the duration of Vitamin-A deficiency (Fig. 1, Table I).

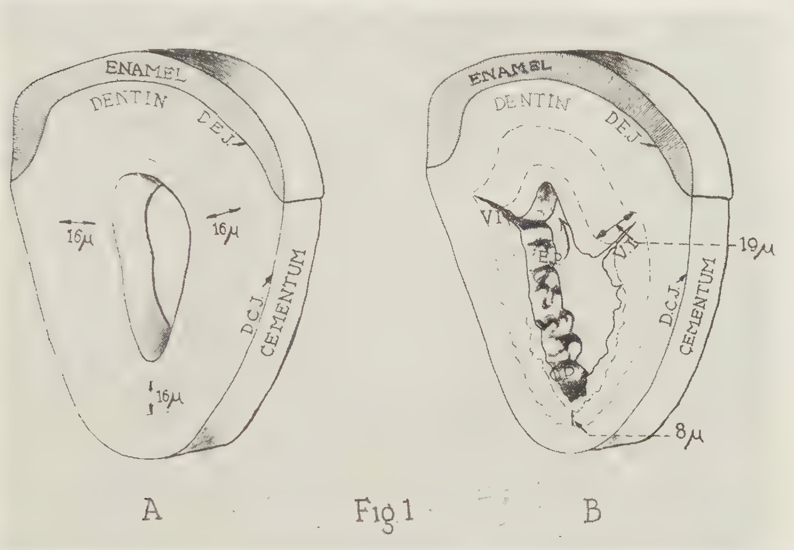

FIG. 1.

A. Tracing and reconstruction of a transverse ground section of the lower incisor taken at the level of the mental foramen of a normal control rat. This animal was injected with alizarine Red S on the 50th and 60th days of life and sacrificed on the 65th day. The alizarine effects (dotted lines) are parallel to the pulpal wall and dentino-enamel, D.E.J., and dentino-cemental, D.C.J. junctions. × 31.

B. Similar field of a rat with same history as in A but placed on vitamin-A deficiency when 21 days old. The enamel-covered dentin is increased and the cementum-covered dentin is decreased in width. The pulpal outline is distorted and narrow at E.P. and wide at C.P. V.I., vascular inclusions. Note alterations in daily appositional rate. × 37.

The disturbances in the rate of appositional growth are manifest between 9 and 19 days following institution of Vitamin-A deficiency (Table I), long before the cytologic and histologic changes become apparent.[2] The final width of the dentin is due primarily to the differences in the rate of growth.

Suboptimal replacement therapy of 4 days' duration did not produce a measurable change in appositional rate. However, full replacement therapy had an immediate effect, causing the normal rate of apposition to be resumed within a period of one to 5 days (Table I).

*Summary.* The rate of apposition of dentin is selectively altered in Vitamin-A deficiency, while the life span of the formative cells is not affected. The rate of apposition is accelerated in the enamel-covered portion and decelerated in the cementum-covered portion. The findings indicate the delicate response of the rate of dentin apposition to Vitamin-A deficiency and suggest the possible use of this reaction as a biologic method of measuring the Vitamin-A content of foods.

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[2] Wolbach, S. B., and Howe, P. R., *Am. J. Path.*, 1933, **9**, 275.

### 10236

## A Pneumonia-Producing Filtrable Agent from Stock Mice.*

F. B. Gordon, Gustave Freeman and J. Marion Clampit. (Introduced by G. M. Dack.)

*From the Department of Bacteriology and Parasitology and the Department of Medicine, University of Chicago.*

A virus causing respiratory disease in stock mice has been reported by Dochez, Mills and Mulliken.[1] We have encountered a similar infectious agent, have made a preliminary pathological study of it, and have attempted to compare it with the Dochez virus and with other agents of disease found in stock mice. The chief purpose of this report, however, is to call attention to the ease with which the disease caused by this agent may be confused in mice with infection due to influenza virus.

Pharyngeal washings from patients with a variety of illnesses (common cold, atypical pneumonia, influenza-like illness) were inoculated intranasally into mice which were sacrificed on the sixth day. The lungs were removed aseptically, emulsified, and passed intranasally to normal mice which were in turn sacrificed, the series being continued indefinitely. As controls, serial passages were made from mice inoculated intranasally with pharyngeal washings from normal persons, with broth, and with normal mouse lungs. In every instance, after a varying number of transfers, lung lesions resembling those produced by influenza virus began to appear in the mice. Their initial appearance varied from the first to the ninth passage and they became maximum in 2 or 3 further passages, killing the mice usually on the third or fourth day. White mice purchased in the open market and weighing 5 to 10 g have been used exclusively, and lung

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* This work was supported in part by a grant from the Fenger Memorial Fund.

[1] Dochez, A. R., Mills, K. C., and Mulliken, B., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 683.

lesions have appeared in mice from 2 different dealers. The appearance of the mice before death and the macroscopic appearance of the lung lesions were the same in all mice, irrespective of original source of inoculum, indicating that the infectious agent was present in the stock mice.

The first signs of illness in the mice are a ruffled coat, a hunched posture and a decrease in food consumption. As the infection progresses, their movements become sluggish, respiration may be labored, and they finally become moribund and die. No mice have survived when once their appearance indicated that infection had occurred. Examination of mice sacrificed at various intervals after inoculation shows that the lungs are first involved at the apices and the dorsal portions. The affected parts are sharply demarcated from the remaining normal lung and have a greyish-pink color. The infection then extends to involve practically all lung tissue and at death the lungs are of a uniform dark red color.

The microscopic lesion in the lungs is characterized by diffuse interstitial pneumonia consisting almost entirely of mononuclear leukocytes and an occasional polymorphonuclear cell. The distribution is generally patchy, but the patches become confluent. The bronchiolar epithelium is well preserved and the lumens occasionally contain exudate composed of mononuclear and polymorphonuclear leukocytes. Although the pneumonic changes are practically identical with those produced by influenza virus in mice, the characteristic desquamation and metaplastic replacement of the bronchiolar epithelium seen in influenza[2] is absent in this diesase. In the liver, focal necrosis of the hepatic cells occurs without other significant inflammatory changes. This further differentiates the disease from influenza in which the liver is usually undisturbed. The infectious agent is present in livers of infected mice as demonstrated by intranasal inoculation.

Blood agar plate cultures have been made routinely of lung emulsions used for inoculation. During the first passages such cultures incubated aerobically and anaerobically almost uniformly showed no growth. Since then bacterial growth has occurred oftener and is due perhaps to a noticeable decrease in the quality of the stock mice used. Lung emulsions have also been cultured upon chocolate agar plates and in brain broth tubes. All types of bacteria found upon these media and anaerobic blood agar plates also grew upon aerobic blood agar plates. When growth does occur it may consist of gram negative rods, gram negative diplococci, or alpha hemolytic streptococci. Mixed cultures are the rule. The type of organisms and amount of growth appear to be totally unrelated, however, to the presence and severity of the lung infection. Mixtures of the cultivated bacteria have not produced illness in mice following intranasal inoculation. Because of the apparent lack of association with cultivable bacteria filtration tests were made. Berkefeld N filtrates of dilute broth emulsions of lungs have caused the infection in 5 of 9 attempts, Seitz filtrates in 1 of 3 attempts. In each case the filtration was controlled by addition of an emulsion of *Bact. prodigiosum* to the material to be filtered. No filtrates showed growth.

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[2] Straub, M., *J. Path. and Bact.*, 1937, **45**, 75.

After the first observations on this infectious agent had been made it was noted that a previous report by Dochez and his colleagues[1] described an infection which appeared to be quite similar to the one we have encountered. Through the courtesy of Dr. Dochez we were able to inoculate mice with lung tissue from their mice. The results obtained were not different clinically or pathologically from those seen in mice infected with our agent. Although the mice showed severe lung lesions at the first passage and the series was carried through 9 generations, it cannot be stated with certainty whether the Dochez virus was passed or the infectious agent of our stock mice was again encountered. Since convalescent mice are not available and production of an antiserum has been unsuccessful to date, cross-immunity tests have not been possible.

Thus far we have not been able to identify the infection described here with any other of the diseases of stock mice. Intracerebral inoculation of mice, and intracerebral, intranasal and subcutaneous inoculation of guinea pigs has caused no apparent illness, indicating that the virus of acute meningo-pneumonitis[3] and of lymphocytic choriomeningitis are not present. We have never seen the characteristic features of infectious catarrh[4] in our mice and have not found the coccobacillary bodies associated with this disease in emulsions of infected lungs. The apparent independence of this infection from cultivable bacteria indicates it is not due to the *Bacterium influenzæ murium.*[5] Mice have been inoculated intraperitoneally and into the foot pads with lung emulsions without producing apparent disease. Such mice showed no resistance to a subsequent intraperitoneal inoculation of ectromelia virus.

Because of the marked resemblance of this infection to that produced by influenza virus in mice we have attempted to make as close a comparison of the two as possible. Four mice, 1 month convalescent from influenza virus (Chicago strains), were inoculated intranasally with a regular passage emulsion of our infected mouse lungs. There was no evidence of any immunity to the infection. Similar tests with influenza virus in influenza convalescent mice have shown a definite immunity. The converse cross-immunity test has not been done due to lack of convalescent mice. No evidence of *in vitro* neutralization was found using antiserum prepared in the rabbit against the PR 8 strain of human influenza virus. Three human serums (patients supplying original pharyngeal washings) also gave no neutralization.

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[3] Francis, Thos., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

[4] Nelson, John B., *J. Exp. Med.*, 1937, **65**, 833; *ibid.*, 843.

[5] Kairies, A., and Schwartzer, K., *Zentralbl. f. Bakt.*, 1936, **137**, 351.

The present significance of this infection appears to be the possibility of its confusion with influenza virus infection in mice or its interference with other work in which mouse lungs are passed. Slight clinical and microscopical differences may be noted between this infection and that of influenza virus, but in general they are quite similar. Our own experience will illustrate the possible difficulties which may arise. After several months' storage in glycerin, 4 strains of human influenza virus were passed rapidly through 4 generations of mice by intranasal inoculation of lung emulsions. A serum neutralization test was done, using a serum which had previously shown strong neutralizing properties against human influenza. No neutralization occurred in any strain, and our conclusion was that our influenza virus had become contaminated with and possibly replaced by the other agent.

## 10237

## Neurogenic Fever Reduced by Nembutal.*

S. W. RANSON AND GEORGE CLARK.

*From the Institute of Neurology, Northwestern University Medical School.*

Following operations on the brain in the region of the optic chiasma and infundibulum, patients frequently develop a hyperthermia which is difficult to control and is often fatal. Similar sharp rises in temperature have been obtained in cats and monkeys by placing lesions in the anterior part of the hypothalamus or in the preoptic region. These operations were performed under nembutal anesthesia and the hyperthermia did not appear until the depressing effect of the nembutal had worn off after the lapse of several hours. The technic involved, the location of the lesions, the course of the hyperthermia and its significance for an understanding of the mechanism involved in temperature regulation have been discussed elsewhere.[1, 2]

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* Aided by a grant from the Rockefeller Foundation.

This investigation was undertaken to study the course of the hyperthermia, when ether instead of nembutal was used as an anesthetic, and the effect of nembutal when injected after a high temperature had been developed. Under ether, lesions were placed bilaterally in the anterior part of the lateral hypothalamus and immediately following the operation the cat was strapped on its side in a comfortable hammock and a continuous record was taken of rectal temperature for the next 20 hours with a Leeds and Northrup Micromax.

Seven out of 11 cats showed postoperative hyperthermia. In one the rectal temperature rose to a high of 104.3° in 450 minutes and in another to 104.8° in 670 minutes. In these 2 cats no nembutal was given. Five others in which the temperature rose more sharply were given nembutal (14 mg per kg) after the temperature had reached levels varying from 104.8 to 106.9. The nembutal caused precipitate drops in temperature in all 5 cats varying in extent from 2.4° to 4.4° (Table I). The time during which the temperature was dropping

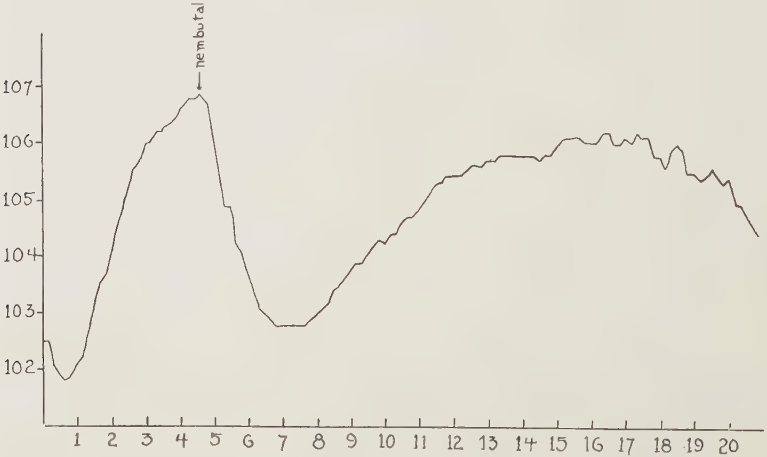

FIG. 1.

Chart showing temperature variations in Cat 132 during the first 20 hours following placing of lesions in the anterolateral part of the hypothalamus. Temperature in degrees Fahrenheit.

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[1] Ranson, S. W., Fisher, C., and Ingram, W. R., *Arch. Neurol. and Psychiat.*, 1937, **38**, 445.

[2] Clark, George, Magoun, H. W., and Ranson, S. W., *J. Neurophysiol.*, 1939.

TABLE I.

Hyperthermia Caused by Anterior Hypothalamic Lesions and Reduction of Fever by Nembutal. Rectal Temperature in Degrees Fahrenheit and Time in Minutes.

| Cat. No. | Min. after operation before injection | Temp. at time of injection | Drop caused by injection of nembutal | Min. from beginning of injection to bottom of fall | High temp. reached after the drop | Min. from bottom of drop to second high temp. |
|---|---|---|---|---|---|---|
| 130 | 280 | 104.8 | 4.4 | 60 | 104.8 | 380 |
| 132 | 280 | 106.8 | 4.0 | 130 | 106.4 | 600 |
| 133 | 360 | 104.9 | 4.2 | 90 | 105.8 | 450 |
| 135 | 280 | 104.8 | 2.4 | 40 | 107.2 | 440 |
| 137 | 720 | 106.9 | 3.5 | 90 | 106.4 | 300 |

varied from 40 to 130 minutes. During this fall the cats slept quietly. As the effects of the nembutal wore off and the cats began to move again the temperature started rising and in several hours reached points as high as or in some cases higher than it was at the time of injection.

Fig. 1 illustrates the rate of rise and fall of temperature in Cat 132. The cat had been on a heating pad during the operation and its temperature fell somewhat before the rise began.

During the sharp rises in temperature following the operation the cats were frequently observed to shiver while their rectal temperatures were as high as 104, 105, or 106°. The cats did not pant and there was not much increase in respiratory rate. The room temperature during these experiments ranged from 79 to 82°.

The dose of nembutal required to produce these sharp drops in temperature was less than half as great as is regularly employed in this laboratory as a surgical anesthetic and it could have been safely repeated when the temperature began to rise a second time. The nembutal was given intravenously in 4 of the cats and subcutaneously in one with substantially the same results. The intravenous injection is made very slowly during a period of 10 minutes. The possibility that this drug might be useful in combating neurogenic hyperthermia in man should receive the consideration of neurosurgeons.

## 10238

# Vasomotor Responses from Application of Drugs to the Medullary Region.

SHIH-CHUN WANG.* (Introduced by S. W. Ranson.)
*From the Institute of Neurology, Northwestern University Medical School.*

Since Cushing[1] reported that injections of pituitrin and of pilocarpine into the human brain ventricles gave similar effects, numerous workers have concerned themselves with the idea that these drugs may give a central action quite different from that following peripheral administration. Many investigators have injected various drugs into almost all parts of the central nervous system, the results, however, were by no means consistent.

Heller[2, 3] reported a negative or small depressor response in cats under urethane anesthesia when 1.5 mg of adrenaline was injected intracisternally, while in dogs a small pressor reaction was obtained with 4 pressor units of pitressin. Histamine (one mg) and acetylcholine (10 r) were reported to be centrally inactive. Haraguchi,[4] on the other hand, found that 1% of acetylcholine or pilocarpine directly applied to the surface of medulla of cats produced a definite depressor response and augmented respiration. The central effect of acetylcholine (0.1-1 r) in cats under paraldehyde or urethane anesthesia was claimed by Dikshit[5] to be inconsistent. According to the recent work of Suh and others[6] on dogs under chloralose anesthesia, 4-40 r acetylcholine given intracisternally or applied on the floor of the fourth ventricle gave an elevation of blood pressure and an augmentation of respiration. The present communication deals with the central action of a group of sympathomimetic and parasympathomimetic drugs on blood pressure and respiration of cats. The cisternal region and the floor of the fourth ventricle were chosen as the sites of administration.

In this series of 17 cats, nembutal (20 mg per kilo) was given to produce a light anesthesia. The cisternum was exposed by removing part of the occipital bone. Usually several applications of drugs were made to the cisternal region before the cerebellum was finally retracted or extirpated to expose the floor of the fourth ventricle.

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* Fellow of the China Medical Board.

[1] Cushing, H., *Proc. Nat. Acad. Sci.*, 1931, **17**, 163.

[2] Heller, H., *Arch. Exp. Path. Pharmakol.*, 1933, **173**, 291.

[3] Heller, H., und Kusunoki, G., *Ibid.*, 1933, **173**, 301.

[4] Haraguchi, S., *Nagasaki Igakkwai Zassi*, 1933, **11**, 191.

[5] Dikshit, B. B., *J. Physiol.*, 1934, **80**, 409.

[6] Suh, T. H., Wang, C. H., and Lim, R. K. S., *Chin. J. Physiol.*, 1936, **10**, 61.

TABLE I.
Varieties of Responses to Different Drugs.

| Drug | Amt. |  | Site | No. of cats |  |  |  | Avg B.P. change in mm Hg among depressor responses |
|---|---|---|---|---|---|---|---|---|
|  | cc | Pressor units |  | Total | Negative | Pressor | Depressor |  |
| Pituitrin | .2 | 2 | IC* | 15 | 2 | 4 | 9 | —15 |
|  |  |  | IVth† | 6 | 1 | 1 | 4 | —13 |
| Pitressin | .2 | 4 | IC | 4 | 1 | 1 | 2 | —18 |
|  |  |  | IVth | 6 | 1 | 1 | 4 | —11 |
| Pitocin | .2 | 0 | IC | 4 | 2 | 1 | 1 | —15 |
|  |  |  | IVth | 5 | 2 | 1 | 2 | —12 |
| Pilocarpine | .2 | .2 mg | IC | 4 | 4 | 0 | 0 | 0 |
|  |  |  | IVth | 9 | 6 | 0 | 3 | —11 |
| Histamine | .2 | .2 mg | IC | 5 | 0 | 1 | 4 | —35 |
|  |  |  | IVth | 7 | 3 | 0 | 4 | —21 |
| Acetylcholine | .2 | 20-100 r | IC | 10 | 3 | 2 | 5 | —15 |
|  |  |  | IVth | 14 | 4 | 3 | 7 | —18 |
| Adrenaline | .2 | .2 mg | IC | 5 | 2 | 1 | 2 | —10 |
|  |  |  | IVth | 9 | 3 | 4 | 2 | —15 |
| Physiological saline solution | .2 | 0 | IVth | 4 | 4 | 0 | 0 | 0 |

*IC—intracisternal application.
†IVth—application to the floor of the fourth ventricle.

The drugs were delivered over its surface with a tuberculin syringe and needle. Mechanical stimulus was carefully avoided, and no 2 applications were made within a single 30-minute interval. Physiological saline solution was occasionally applied as a control. The right carotid artery was cannulated and connected with a mercury manometer for blood pressure and a tracheal cannula connected to a tambour was inserted to record respiratory movements.

From the data, one can see that there is no characteristic difference in response obtained by administration of drugs through these two routes. Among 31 trials with pituitrin and pitressin, an immediate depressor response (11-18 mm Hg) was observed in 19 cases, but when these drugs were injected intravenously, a definite pressor response was obtained. Responses to pitocin and adrenaline were variable, and pilocarpine was rather inactive. All the pressor reactions so far obtained were small and delayed with a period of latency of at least 30 seconds. Since the drugs themselves have a peripheral sympathomimetic action, it is possible that they were slowly absorbed into the blood stream and acted peripherally. Histamine gave a marked depression of blood pressure (21-35 mm Hg) in 8 of the 12 cases. It is of interest that the other parasympathomimetic drug, acetylcholine, also produced a depressor response of 15-18 mm Hg in a large number of cases, but 5 out of 24 trials showed a simple delayed pressor reaction. We were unable to observe a definite vasomotor change with acetylcholine of less than 20 r. The respiration as far as the rate and excursion were concerned showed no conspicuous change throughout this series.

We have no satisfactory explanation for the great divergence of results among our own and other workers. It seems to us that other than the difference in the species of the animal used and the dosage of drugs, the degree and type of anesthesia are of great importance in determining the variability of response.[7] It is possible that the drugs act non-specifically and that pressor or depressor responses are dependent upon the degree of selective depression of the vasomotor centers (pressor or depressor) as a result of the anesthesia.

*Summary.* Small doses of pituitrin, pitressin, histamine and acetylcholine when applied to the cisternal space or the floor of the fourth ventricle of cats under light nembutal anesthesia usually give a small depressor response. The effects of pitocin and adrenaline are inconsistent. Pilocarpine is inactive. There is occasionally an unexplained delayed pressor response with central application of acetylcholine.

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[7] Jackson, D. E., *J. Lab. Clin. Med.*, **1934**, **20**, **1**.

### 10239 P

# Bacterial Flora in Experimental Dental Caries of the Rat.

R. Wendell Harrison. (With the assistance of Elizabeth Hemmens.)

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According to Miller's theory[1] dental caries is caused by acids elaborated by microörganisms. Of the acidogenic and aciduric bacteria present in the plaques covering early lesions and in the carious dentine of advanced cavities, lactobacilli and acid-producing streptococci are the forms commonly believed to be implicated in decalcification of human teeth. However, these organisms are regularly found in the mouths of caries-free rats and if they are involved in experimental rat-caries their activities must be dependent upon other not well understood factors. The present investigation was made with animals used by Dr. B. F. Miller[2] in a study of the inhibition of rat-caries and cultures were taken with his kind coöperation.

Material from the surfaces of molar teeth of 60 young rats was cultured aërobically, at the beginning of the diet periods and again after 7 and 13 weeks, on Kulp's agar,[3] Jay's broth,[4] blood-agar, and brom-cresol-green serum-dextrose agar. The methods were not quantitative but all samples were handled in the same way and the results therefore have comparative value. In the first culture-period streptococci comprised from half to three-fourths of the colonies obtained. They were in general about equally divided between $\alpha$, $\beta$ and $\gamma$ types and in most cases about twice as many were acidogenic as non-acidogenic. The remaining organisms were largely lactobacilli, staphylococci, and micrococci. Other forms were encountered less frequently and usually in small numbers. From Jay's and modified Kulp's media lactobacilli were recovered in all cases but since these media tend to suppress organisms that are not aciduric, comparative counts of acidogenic organisms were confined to indicator-plates.

The studies of Miller[2] have shown a high incidence of cavities in animals on a caries-producing diet, fewer lesions in a group receiv-

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[1] Miller, W. D., *The Microorganisms of the Human Mouth*, Philadelphia, 1890.

[2] Miller, B. F., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 389.

[3] Kulp, W. L., *Science*, 1927, **66**, 512 (Hadley modification: Hadley, F. P., *J. Dent. Res.*, 1933, **13**, 415).

[4] Jay, P., and Voorhees, R. S., *Dent. Cos.*, 1927, **69**, 977, and personal communication.

TABLE I.

Comparative Percentages of Lactobacilli and Acidogenic Streptococci Cultivated from the Tooth-surfaces of Rats on Caries-Producing and Caries-Inhibiting Diets.

| Exp. group | First culture period (7 wk) | | | Second culture-period (13 wk) | | | Caries† at 16 weeks |
|---|---|---|---|---|---|---|---|
| | Total organisms | Acidogenic organisms | | Total organisms | Acidogenic organisms | | |
| | % acidogenic | % lactobacilli | % streptococci | % acidogenic | % lactobacilli | % streptococci | |
| Normal stock* (60 rats) | 52 | 18 | 23 | 52 | 18 | 23 | |
| Caries diet (20 rats) | 35 | 18 | 43 | 36 | 18 | 25 | +++ |
| Caries diet with: | | | | | | | |
| NaF (10 rats) | 24 | <1 | 19 | 51 | 22 | 19 | ± |
| $CaF_2$ (10 rats) | 27 | 1 | 45 | 48 | 14 | 42 | + |
| Iodoacetic acid (10 rats) | — | — | — | 72 | 3 | 90 | — |

*The cultures from the normal stock rats were made at the outset of the experiment and were not repeated. The data are placed in both sets of columns for comparison.

†+++ = high incidence of dental caries, + and ± = low and very low incidence. — = no carious lesions.

ing this diet together with calcium fluoride and little or no caries in groups with added sodium fluoride or iodoacetic acid. Neither the non-acidogenic flora nor the acidogenic flora as a whole showed changes which could be correlated with the development of cavities or with the diets of the animals. Of the acidogenic organisms only streptococci and lactobacilli were regularly found in sufficient numbers to have probable significance. It should be noted that because variations in the amount of inoculum colony-counts cannot here be compared directly and percentages are therefore used. As shown in Table I streptococci varied erratically from 19% to 45% of the total acidogenic flora and reached 90% in the iodoacetic acid group which had no caries. The data indicate no relationship between the presence of acidogenic streptococci on the tooth surface and the development of caries. Lactobacilli comprised 18% of the acidogenic organisms at the beginning of the experiments and remained at that level in the caries-diet group. However, they were reduced to 3% in the final cultures from the group on iodoacetic acid and to 1% in the cultures at 7 weeks from the groups on the fluoride-diets. These reductions, although temporary in the fluoride-groups, may be of possible significance with respect to the failure of caries to develop in these animals.

After 16 weeks 10 carious and 10 non-carious teeth were removed, carefully cleaned with alcohol and sterile water, ground with mortar and pestle, and cultured. The average bacterial count of 9 non-carious teeth, omitting one specimen with a high staphylococcus-count, was only 28 per mg. The flora in general was the same as in swab-cultures from normal rats' teeth, about 30% of the organisms being acidogenic streptococci.

The total bacterial counts from 10 carious teeth averaged about 500 per mg. About 60% of the organisms were acidogenic streptococci. Lactobacilli were found in small numbers in cultures from only 6. The other organisms had about the same relative distribution as in the swab-cultures of the teeth *in situ*.

The very low incidence of lactobacilli in cultures from carious teeth suggests that they may not be involved in the advanced carious process. On the other hand, the high proportion of acidogenic streptococci might be taken as evidence of some relationship to the lesion. Data obtained from human teeth,[5] indicating that lactobacilli may be involved in early lesions of the enamel, and that cocci may predominate in carious dentine, are in a measure substantiated by the findings in this investigation.

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[5] Blayney, J. R., personal communication.

### 10240

## Reactions of Hydrogen Selenide with Hemoglobin Derivatives.

Robert D. Barnard and William Neitzel. (Introduced by A. J. Carlson.)

*From the Chemistry Laboratory of the Northern Illinois College.*

Keilin[1] reported the formation of a compound between hydrogen sulfide and ferrihemoglobin which appears to come into the category of a class of ferrihemoglobin compounds, the existence of which was predicted by Gamgee[2] and termed "hemoferrides" by Barnard.[3] Because of the close chemical, physical and toxicologic similarities of hydrogen sulfide to hydrogen selenide, we have studied the effects of the latter on various hemoglobin derivatives. The analogy between the two gases appears to extend to their behavior toward hemoglobin solutions, thus, treatment of ferrihemoglobin solutions with hydrogen selenide results in the formation of a compound which is spectroscopically similar to hydrogen sulfide ferrihemoglobin whereas the ferrihemochromogens are reduced by hydrogen selenide to the corresponding ferrohemochromogens.

*Experimental. I. Preparation of Solutions.* 1. *Ferrihemoglobin.* Fresh erythrocyte suspensions (rat, dog, and human) of known oxygen capacity were hemolyzed with ether-saturated water and diluted to a concentration of 0.1 millimolar in terms of iron molarity. An equivalent of potassium ferricyanide was added and the solution kept at room temperature for an hour before use. Complete oxidation to ferrihemoglobin was confirmed by spectroscopic examination.

2. *Ferrihemochromogens.* 63 mg of crystalline ferriheme chloride was dissolved in each of the following solvents: (a) 5% aqueous pyridine to form *pyridine ferrihemochromogen hydroxide.* (b) 26% ammonia water to form *ammonia ferrihemochromogen hydroxide.* (c) 2% sodium cyanide to form *cyanoferrihemochromogen cyanide.*

3. *Ferrihemes.* 63 mg of crystalline ferriheme chloride was dissolved in (a) 1% sodium carbonate to give *sodium ferriheme hydroxide* and (b) 3% triethanolamine to form *triethanolamine ferriheme hydroxide* solution. All ferrihemochromogen and ferriheme

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[1] Keilin, D., *Proc. Soc. B.*, 1933, **113**, 393.

[2] Gamgee, A., in Schafer, E. A., *A Textbook of Physiology*, Edinburgh, 1898, 242.

[3] Barnard, R. D., *J. Biol. Chem.*, 1937, **120**, 177.

solutions were 0.2 millimolar, a concentration that is well suited for spectroscopic work.

II. *Treatment of Samples with Hydrogen Selenide.** 5 cc of the sample to be studied was placed in each chamber of a double spectroscopy vessel. The outlet tube of the hydrogen selenide generator was immersed in one of the chambers and the gas ejected through the solution at the rate of 3 bubbles per second for 10 seconds. The saturated sample was then examined through a Hilger constant deviation angle spectrometer along with the control sample which had not been subjected to the action of the gas.

The spectroscopic observations were also controlled by an examination of colloidal aqueous selenium, sodium polyselenide solutions and the reaction product of hydrogen selenide on potassium ferricyanide solution. All of these products are red in color but show no definite absorption bands in the visible spectrum.

*Results.* Hydrogen selenide immediately converts ferrihemoglobin solution into one of a salmon red color. The change is apparent in less than 2 seconds by the method of saturation employed. The solution shows a single broad absorption band between 5400 Å and 5200Å.

On treatment with a drop of saturated sodium hydrosulphite solution this band disappears, to be replaced by that of hemoglobin. Since ferrihemoglobin is the direct precursor of hemoglobin (Conant[4]), this may be taken as evidence of the reversibility of the reaction between hydrogen selenide and ferrihemoglobin. Hydrogen selenide appears to be without effect on pyridine ferrihemochromogen hydroxide insofar as compound formation is concerned. The solution turns red after 10 seconds of saturation but spectroscopically it cannot be distinguished from the original substance. Cyanoferrihemochromogen cyanide and ammonia ferrihemochromogen hydroxide are immediately reduced by hydrogen selenide to the corresponding ferrohemochromogens and, on exposure to air, these latter are soon reoxidized to the original ferrihemochromogens. The cyanide and ammonia solutions are strongly alkaline; the pyridine solution is not; the effect in the strongly alkaline solutions is probably that of alkali selenide and is entirely comparable to the effect of alkali sulfide on the same solutions.

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* Hydrogen selenide was prepared by hydrolysis of aluminum selenide. 4 g of electrolytic aluminum and 10 g of powdered black selenium are intimately mixed in a mortar and transferred to a pyrex test tube over a small pellet of metallic sodium. The tube is heated over a Bunsen flame until the contents fulgurate. The upper portion of the contents is removed discarding the sodium selenide which remains in the bottom. About 5 g of granulated aluminum selenide is placed in a small wash bottle, the inlet tube of which is connected to a tank of nitrogen. When the latter is released it carries over to the outlet an appreciable (and dangerous) concentration of hydrogen selenide which is liberated by the small quantity of moisture present.

[4] Conant, J. B., *J. Biol. Chem.*, 1923, **57**, 401.

Passage of hydrogen selenide through the sodium ferriheme hydroxide solutions causes a change from the greenish color of these solutions to salmon red. The absorption band of the alkaline ferriheme disappears and is replaced by a single band in the yellow centering at 5620Å. Since alkaline ferriheme is irreducible by the sulfides in the absence of suitable nitrogenous "hemochromogen formers," (Bertin-Sans and deMontessier[5]), and since there is no evidence of the characteristic hemochromogen doublet in the reaction product of hydrogen selenide and alkaline ferriheme, the latter probably represents a hemoferride.

From some studies not as yet published, we have found that solutions of hematin in triethanolamine are ferrihemes, the nitrogen of the amine being incapable of coördination with the iron. The results in this study confirm this conclusion. Triethanolamine solutions of hematin behave toward hydrogen selenide in a manner similar to sodium ferriheme hydroxide; the band in the red is replaced by one in the yellow green. This latter band is not so well defined as that appearing in the sodium carbonate solutions of hematin and may possibly represent a slight tendency toward the formation of a hemochromogen.

Hydrogen selenide is without effect on the spectroscopic bands of either oxyhemoglobin or alkaline hematoporphyrin. On solutions of methemoglobin in 0.5% acetic acid, hydrogen selenide reacts with the formation of a greenish pigment similar in appearance to the decomposition products which arise from hemoglobin by the prolonged action of sulfide and described by Hoppe-Seyler[6] and Araki.[7]

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[5] Bertin-Sans, H., and deMontessier, J., *Compt. rend. Acad.*, 1892, **114**, 923.

[6] Hoppe-Seyler, F., *Zentralbl. f. med. Wissensch.*, 1863, **28**.

[7] Araki, T., *Z. physiol. Chem.*, 1890, **14**, 405.

## 10241 P

# Immediate and Delayed Disposal of Acacia Injected Intravenously in the Dog.

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The intravenous injection of acacia is followed by a decrease in the concentration of the serum proteins including fibrinogen, a diminution in the total circulating protein,[1-5] and alterations in the colloidal osmotic pressure.[7] The acacia is stored largely in the liver.[2, 6] These results have been attributed to an interference with the proteogenic function of the liver.[2]

Our experience has shown that diets containing 50 cc of codliver oil, 150 cc tomato juice, 200 g of meat, and 200 g of prepared dog food are adequate[8] and maintain blood proteins at a high level over a period of a year's time.

When acacia solutions (12% acacia in 0.85% sodium chloride) approximating the plasma volume are injected, there is an immediate increase in blood volume (22 to 30%), a decrease in serum proteins (both albumin and globulin) and an increase in the acacia. The values for serum acacia plus protein equal the original value for serum protein. One and one-half hours after the injection the blood vessels of the viscera are engorged, ascitic fluid has accumulated and there is free bleeding from the incision. Of the injected acacia (125 g) the major part is in the muscles (54 g) and serum (48 g); lesser amounts in urine (9.0 g), ascitic fluid (7.18 g), liver (4.46 g) and kidneys (1.9 g). The large amount in the muscles is due to vascular dilatation, and the ascites to an "emergency weeping" into the peritoneal cavity.

When an animal is repeatedly injected (Table I) with allowance for recovery, there is a repetition of the phenomena occurring after

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[1] Dick, M. W., Warweg, E., and Andersch, M., *J. A. M. A.*, 1935, **105**, 655.

[2] Heckel, G. P., Erickson, C. C., Yuile, C. L., and Knutti, R. E., *J. Exp. Med.*, 1938, **67**, 345.

[3] Hall, W. Knowlton, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 46; *Am. J. Physiol.*, 1938, **123**, 88.

[4] Erickson, C. C., Heckel, G. P., and Knutti, R. E., *Am. J. Path.*, 1938, **14**, 537.

[5] Lepore, M. J., *Ann. Int. Med.*, 1937, **11**, 285.

[6] Andersch, M., and Gibson, R. B., *J. Pharm. and Exp. Therap.*, 1934, **52**, 390.

[7] Onozaki, Nobuske, and Sanada, Yukikazu Tohoku, *J. Exp. Med.*, 1935, **25**, 120.

[8] Halman, Russell L., Mahoney, Earle B., and Whipple, George H., *J. E p. Med.*, 1934, **59**, 269.

TABLE I.
Repeated Intravenous Injections of Acacia.

| Date | Acacia |  |  | Serum proteins, g% |  |  | Serum acacia g | Serum acacia plus protein | Osmotic pressure mm of water |
|---|---|---|---|---|---|---|---|---|---|
|  | Amount given previously g | Interval since last injection | Amt | Alb. | Glob. | Total |  |  |  |
| 4/21 | 232 | 2 mos. |  | 4.30 | 2.05 | 6.35 | 0.63 | 6.98 | 306.8 |
| 4/22 | 288 | 30 min. | 56 |  |  | 3.61 | 4.17 | 7.78 | 453.8 |
| 4/26 | 288 | 4 days |  |  |  | 4.45 | 2.73 | 7.18 | 391.1 |
| 4/28 | 288 | 30 min. | 58 |  |  | 2.48 | 4.81 | 7.29 | 423.4 |
| 5/4 | 346 | 6 days |  |  |  | 4.00 | 2.50 | 6.50 | 390.4 |
| 5/11 | 404 | 30 min. | 58 |  |  | 2.13 | 4.69 | 6.82 | 422.3 |
| 5/17 | 404 | 6 days |  | 2.25 | 1.35 | 3.60 | 2.59 | 6.19 | 399.0 |
| 5/18 | 462 | 30 min. | 58 |  |  | 2.07 | 4.78 | 6.85 | 421.4 |
| 5/24 | 462 | 6 days |  | 2.25 | 1.16 | 3.41 | 3.26 | 6.67 | 323.0 |
| 5/25 | 520 | 30 min. | 58 |  |  | 2.03 | 5.77 | 7.80 | 431.5 |
| 5/31 | 520 | 6 days |  |  |  | 3.21 | 2.82 | 6.03 | 357.8 |
| 6/16 | 520 | 16 days |  |  |  | 3.57 | 1.92 | 5.49 | 258.5 |

a single injection. Immediately after an injection there is also an increase in colloidal osmotic pressure with a return of it to normal later. The fall in pressure is paralleled by an increase in serum proteins and a decrease in serum acacia.

The acacia is progressively deposited in the liver as shown by periodic biopsies, gradual increase in liver size and tissue analyses. In one animal the liver was 5 times normal size. Of the 756 g injected, 444.9 g (58.8%) were recovered from the liver, 105.4 g (13.9%) from the muscle, 41.1 g (5.4%) from the serum, and 11.4 g from other sources. This left a balance of 153.2 g for excretion in the urine.

The blood is cleared fairly rapidly of acacia, largely through storage. The mobilization from storage is small but definite, as shown by its presence (1%) in the serum one year after last injection. The recession of serum proteins with the addition of acacia to the blood and their return with the storage of acacia indicate a reciprocal functional relationship between the two. The rise of the colloidal osmotic pressure with the injection of acacia and its fall with storage of acacia may now receive a valid explanation. Obviously the adjustments are directed towards the maintenance of a constant osmotic pressure.

Hence, under the experimental conditions cited, the diminution of the serum proteins is not to be regarded as an index of failure of the proteogenic function of the liver, but as due to a displacement of the proteins by the acacia.

## 10242

## Effect of Testosterone Propionate on Glycogen Content of Human Vaginal Smears.

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As part of an investigation into the subject of the hormonal control of the glycogen present in the vaginal mucosa, it was felt desirable to determine whether glycogen could be demonstrated in human vaginal smears. This has been accomplished by the following method:

*Technic of Glycogen Stain.* Patients are instructed to take a plain water douche the evening before the smears are taken. A speculum is inserted into the vagina and the cervix and fornices are wiped clean. The speculum is then withdrawn, at the same time sweeping off the secretion from the anterior wall with the posterior blade of the speculum. The secretion on the blade is then diluted with an equal part of normal saline and spread on a glass slide. Fixation is obtained by drying in air. A modification of the Best carmine[†] stain (which is used in the Department of Pathology of the Mt. Sinai Hospital to demonstrate glycogen in tissues) was adapted for the smears.[‡]

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* Joseph Brettauer Fellow in Gynecology.

1. Stain with hematoxylin--5 min.
2. Rinse in cold water.
3. Stain with carmine solution—15 min.

Stock carmine is made as follows:

| | |
|---|---|
| Carmine | 1 |
| Pot. chloride | 2.5 |
| Pot. carbonate | .5 |
| Distilled water | 50 |

Mix and boil 1 minute until solution turns a deep red. Cool at room temperature. Add 10 cc of ammonium hydroxide. Keep over night in the ice box before using.

*Carmine solution for use*—prepared fresh.

| | |
|---|---|
| Stock solution, filtered | 10 cc |
| Ammon. hydroxide | 15 |
| Absolute methyl alcohol | 10 |

4. Differentiate in

| | |
|---|---|
| Absolute ethyl alcohol | 16 |
| ’’ methyl ’’ | 8 |
| Distilled water | 20 |

Immerse 4-5 times

5. Absolute alcohol—2 immersions
6. Xylol—2 immersions
7. Mount with balsam

The glycogen appears as deep red granules in the cytoplasm. In some cells the granules are coarse, while in others they are so fine that the cytoplasm is diffusely pink. The nuclei stain blue. This stain also reveals distinctly the morphologic characteristics of the cellular elements in the smear.

With the aid of this method, studies are being carried on to determine the glycogen content of the vaginal smears in hypo-ovarian states, in menopause, in castrates and in senile states. At present we wish to report on the effect of testosterone propionate on the glycogen content of the smears of normally cyclical women.

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[†] *Pathological Technique,* Mallory and Wright, 8th Edition, 1924.

[‡] We wish gratefully to acknowledge the helpful advice of Drs. Paul Klemperer and Sadao Otani in the adaptation of the glycogen stain to the vaginal smear.

Smears taken throughout the menstrual cycle (except during the period of actual menstruation) in women with regular cycles show slight variations in glycogen content which are difficult to interpret. In some women the smears are uniformly lower in glycogen content than in others. This phase of the problem will be reported on later. For the investigation reported here 2 young women, in whom abundant glycogen had been demonstrated during a preceding cycle, were given testosterone propionate to determine its effect on the glycogen content of the epithelial cells. The following is a protocol of Case I:

Patient N.F. Age 25. Regular 28-day cycle. Preliminary smears showed abundant glycogen. Testosterone propionate injections were started on the 1st day of the cycle and continued to the 18th day. Single doses varying from 25-100 mg were given intramuscularly every other day until a total of 700 mg was given. No changes were noted in the smear until after the 9th day when 500 mg had been given. On this day, the smear contained a scattering of small, oval epithelial cells (atrophy cells—"deep cells" of Papanicolaou and Shorr[4, 8]) abundantly supplied, however, with glycogen. From this day the vaginal smears showed gradual disappearance of the squamous epithelial cells containing the glycogen and their replacement by the atrophy cells. After a few days, the atrophy cells grew progressively smaller in size, fewer in number and poorer in glycogen content. A further significant feature was the appearance of leucocytes in increasing numbers. From the 18th to the 25th day of the cycle, the vaginal smear consisted of a predominance of leucocytes with a scattering of very small atrophy cells. Squamous epithelial cells and glycogen were absent in the smears during this period. (The menstrual period which was expected at the 28th day did not appear.) Signs of beginning restoration to normal began to appear after the 25th day of the cycle. Small, round epithelial cells showing traces of glycogen began to appear among the leucocytes. During the next 10 days, rapid progressive changes were manifested both in the morphologic characteristics as well as in the glycogen content from day to day. There was a rapid increase in the number of round and oval epithelial cells containing glycogen and a parallel decrease in the number of leucocytes. Within a few days these atrophy cells were replaced by epithelial cells which appeared to grow larger from day to day and contained increasing amounts of glycogen. On the 35th day, the smear consisted of large, clearly-defined, squamous epithelial cells abundantly supplied with glycogen. No atrophy cells and no leucocytes were present. This status per-

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[8] Papanicolaou, G. N., Ripley, H. S., and Shorr, E., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 689.

sisted until the 54th day of the cycle when the patient began what appeared to be a normal period lasting 4 days. Essentially similar changes were noted in the second case.

*Summary.* A method of demonstrating simultaneously the presence of glycogen and the morphologic characteristics of the desquamated cellular elements of the vaginal mucosa is presented. The glycogen in the desquamated vaginal epithelial cells of normally menstruating women can be made to disappear by administering adequate amounts of testosterone propionate. The disappearance of the glycogen is apparently dependent upon the production of atrophic changes in the vaginal mucosa since the first effect of the testosterone propionate is the disappearance of the squamous epithelial cells and their replacement by cells from the deeper layers of the mucous membrane. Coincident with this change in the size of the cells, the glycogen begins to decrease steadily and finally vanishes completely. Restoration of the glycogen in the smears parallels closely the reappearance of the normal vaginal epithelium.

The question arises as to the mechanism of this regression in the smear and the disappearance of the glycogen. There is experimental evidence indicating that androgens negate the biologic effect of estrogens.[1-3] It is, therefore, conceivable that the testosterone propionate in the cases reported here inactivated the estrogen formed in the individual and, as a result, atrophy of the vaginal mucosa occurred with loss of cornification and consequent disappearance of glycogen. It seems likely, however, that the regressive changes induced in the smear are also the end results of inhibition of the gonadotropic hormone formation of the hypophysis resulting in suppression of the follicular ovarian cycle. That the testosterone propionate probably inhibits the hypophysis is suggested by the following observations: (a) the excessive gonadotropic hormone excretion in a human female castrate can be suppressed with testosterone propionate;[5] (b) ovulation can be similarly inhibited in monkeys;[6] (c) menstruation can be inhibited and the estrogen and progesterone effects in the endometrium of cyclical human females can be suppressed by administering adequate amounts of testosterone propionate.[7]

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[1] Ihrke, I. A., and D'Amour, F. E., *Am. J. Physiol.*, 1931, **96**, 289.

[2] Robson, J. M., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 49.

[3] Browman, L. G., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 205.

[4] Papanicolaou, G. N., and Shorr, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 585.

[5] Salmon, U. J., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 488.

[6] Zuckerman, S., *Lancet*, 1937, **2**, 676.

[7] Gaines, J. A., Salmon, U. J., and Geist, S. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 779.

### 10213

# The Effect of Glucose on the Oxidation of Alcohol *in vitro.*

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*From the Psychiatric Divisions, Kings County Hospital and Bellevue Hospital, New York.*

The metabolism of alcohol has been the subject of numerous recent publications in which it has been suggested that the oxidation of alcohol may be catalyzed by the simultaneous oxidation of carbohydrate. The ingestion of food (Southgate[1]) or glucose (Bornstein and Loewy[2]) along with alcohol resulted in a lower blood concentration of alcohol. Carpenter and Lee[3] found that after the ingestion of glucose or fructose with alcohol, the alcohol was present in the expired air for a shorter period. Clark and Morrissey[4] measured the rate of disappearance of alcohol from the blood of dogs given doses of 3 g per kilo. They found that the administration of insulin and glucose accelerated the disappearance of alcohol. More recently Clark, Morrissey and Fazekas[5] have shown that the liver of depancreatized cats could not oxidize alcohol in the Warburg apparatus. In this respect the metabolism of alcohol in the body resembles that of the acetone substances. Shaffer[6] showed that the oxidation of acetoacetic acid in alkaline solution by hydrogen peroxide was accelerated when glucose was simultaneously oxidized. We are here reporting similar experiments on the effect of glucose on the *in vitro* oxidation of alcohol.

The control solution contained 100 mg % of alcohol, 20 milliequivalents percent of hydrogen peroxide, 1% sodium hydroxide, and 1 mg % ferric ammonium citrate. The experimental solution contained 1% glucose in addition. The reaction was studied at 25°C. Alcohol analyses were made at various intervals during the following 24 hours by the macro-method of Abels.[7] The accuracy of the method was estimated from a series of analyses of 39 known solutions ranging from 30 to 110 mg %, in which the average error was 5.1 mg %. In 34 instances the error was less than 10 mg %, while in the remaining 5 it was 15 mg %.

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[1] Southgate, H. W., *Biochem. J.*, 1925, **19**, 737.

[2] Bornstein, A., and Loewy, A., *Biochem. Z.*, 1927, **191**, 271.

[3] Carpenter, T. M., and Lee, R. C., *J. Pharm. and Exp. Therap.*, 1937, **60**, 264, 286.

[4] Clark, B. B., and Morrissey, R. W., *Am. J. Physiol.* (proc.), 1938, **123**, 37.

[5] Clark, B. B., Morrissey, R. W., and Fazekas, J. F., *Science*, 1938, **88**, 285.

[6] Shaffer, P. A., *J. Biol. Chem.*, 1921, **47**, 433.

[7] Abels, J. C., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 346.

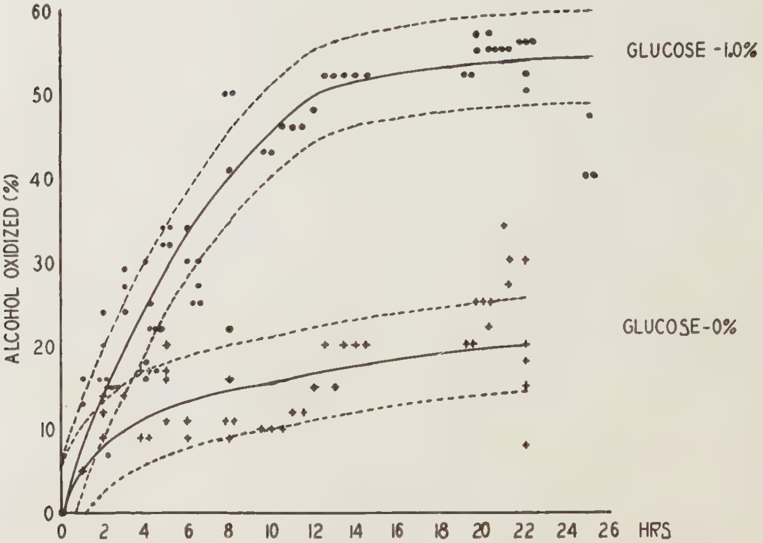

Fig. 1.

The effect of glucose on the oxidation of alcohol *in vitro*.

The data are presented in Fig. 1. The dotted lines enclose an area about each curve equal to plus and minus the estimated average error of the analytical method, 5.5 mg %. It may be seen that the large majority of the points fall within these areas. The curve for the rate of oxidation of alcohol appears to approach a maximum after 12 hours, and we have analyzed this maximum statistically. The average amount of alcohol oxidized in the control solution was 22 mg %, with a probable error of ±0.97. When glucose was present in solution the average amount oxidized was 52 ± 0.67 mg %. The difference with its probable error was 30 ± 0.60. This statistically significant difference indicates that the presence of glucose resulted in a marked acceleration of the oxidation of alcohol. We are unable to explain this apparent catalytic effect of glucose on the oxidation of alcohol *in vitro*.

### 10244

## Sputum Studies in Lobar Pneumonia. Phagocytosis and the Effect of Serum Therapy.

ARTHUR W. FRISCH.* (Introduced by W. O. Nelson.)

*From the Department of Bacteriology, College of Medicine, Wayne University, Detroit, Mich.*

This laboratory is beginning a thorough study of sputum from cases of lobar pneumonia in the hope that the recognized cyclic changes which occur may eventually be correlated with the clinical condition of the patient and thereby become an aid in the therapy and prognosis of this disease. The present communication deals with a few of the microscopic findings which seem to be significant. To the author's knowledge the data herein described have not as yet been recorded in the literature. Most of the sputum studies in lobar pneumonia were made long before the discovery of the various pneumococcal types and are best summarized by von Hoesslin[1] and Clifford.[2]

*Methods.* The lobar pneumonias included in this series of more than 60 cases were selected in the sense that only typical ones were followed. Thin smears were made directly from representative portions of rusty sputum and treated with Wright's blood stain. Sputum examinations were made at 4- to 12-hour intervals so that 2 or more daily specimens were obtained from each patient until recovery or fatal termination.

The capsules of the pneumococci usually did not stain but were readily visualized as clear or occasionally, when stained, as pink zones around the dark blue diplococci. The size of the capsule corresponded fairly well to that noted by means of the "quellung" reaction and in all cases where no capsules were demonstrable in the stained slide the organisms were not directly typeable. Both the capsules and cell bodies of the pneumococci varied greatly in size, shape and affinity for the Wright stain in the same and different cases infected with a similar type of pneumococcus.

An attempt was made to place the data on a quantitative basis by counting the number of diplococci in 50 or more phagocytizing leu-

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* This study was partially aided by a grant from the Commonwealth Fund to the Michigan State Laboratory of Health. Particular thanks are due Doctors A. E. Price, H. L. Clark, G. B. Myers, O. A. Brines and Mrs. Miriam Altshuler for their criticisms, encouragement and assistance.

[1] Hoesslin, H. von, Julius Springer, Berlin, 1926.

[2] Clifford, R., McMillan Co., New York, 1932.

kocytes. Frequently it was difficult to distinguish between disintegrating microörganisms and toxic granulation; therefore the counts included only those diplococci with intact structure and definitely appearing to be in the cytoplasm of the phagocyte.

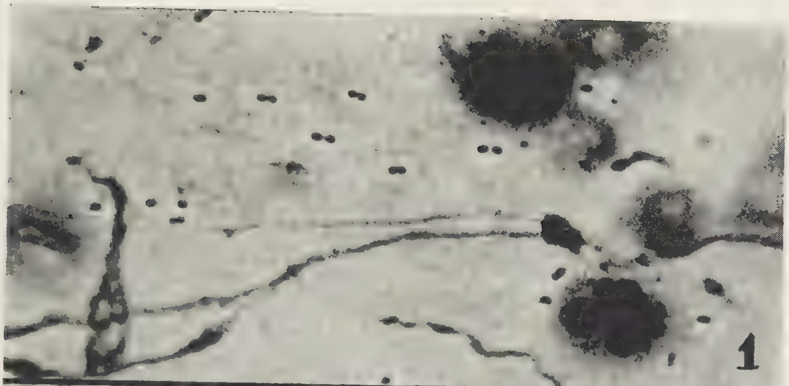
*Microscopic Findings.* As has been pointed out by Clifford the phagocytes tend to be more active as the pneumonia progresses. This observation was confirmed by direct counts in some 23 cases of lobar pneumonia. The average number of diplococci per phagocyte in the admission sputa was 5 with variations ranging from 2 to 10. The highest average number at any time during the subsequent course of the disease was found to be 8 with variations ranging from 3 to 16. While this total increase in phagocytosis might be of value in an individual case or even become statistically important when sufficient cases were studied, it was felt that the number of a particular kind of pneumococcus within the cytoplasm of the phagocyte might be even more significant. For this reason the phagocytosis has been divided into 3 types depending upon the appearance of the organisms within the leukocyte. At present these will merely be described and no attempt will be made to correlate them with the clinical condition of the patient.

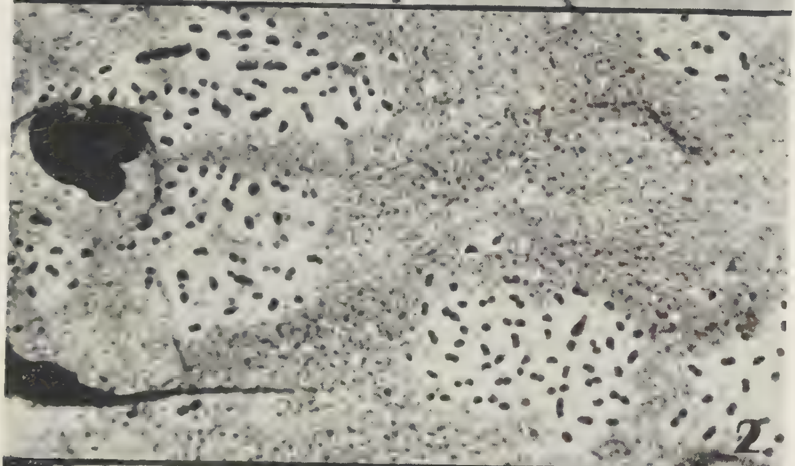
In the first type of phagocytosis, as illustrated in Fig. 3, the pneumococci within the cytoplasm of the leukocyte are definitely encapsulated. In patients who did not receive serum the number of capsulated diplococci per phagocyte varied from 1 to 5.

A second kind of phagocytosis, as illustrated in Fig. 4, is one in which the capsule is not demonstrable with the Wright stain, but its presence is suggested by the fact that the diplococci within the phagocyte are isolated from each other. Considerably larger numbers of such organisms were phagocytized and while an average of 10 per leukocyte was high, one could find as many as 25 within a single phagocyte.

In the third type of phagocytosis, as illustrated in Fig. 5, clumps of uncapsulated organisms may be seen within the cytoplasm of the leukocyte. The polymorphonuclear cells seem to be able to engulf these diplococci with ease and frequently a single phagocyte contained as many as 25 to 50 such organisms.

Because 40 of the cases of lobar pneumonia studied received serum at some time during their illness, it was possible to follow the effect of the treatment on the pneumococci present in the sputum. Capsulated pneumococci in sputum obtained before therapy was instituted, were evenly dispersed throughout the smear and each diplococcus was isolated from others present in the same field. This is well illustrated in Fig. 1. The first effect of horse or rabbit anti-





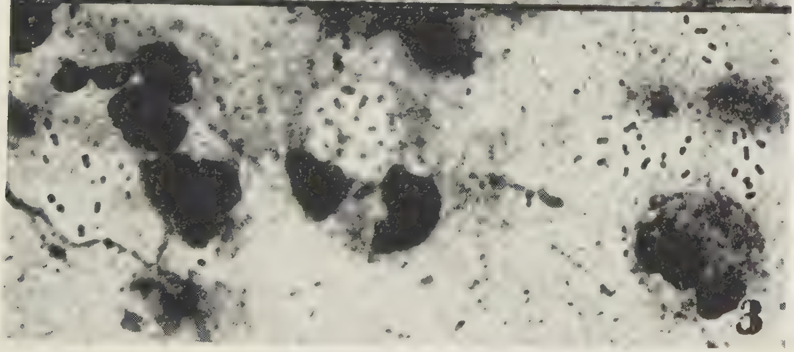
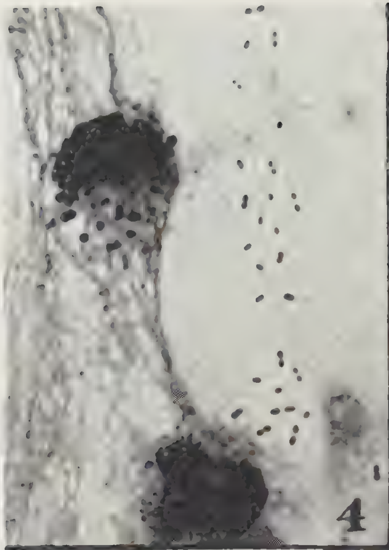
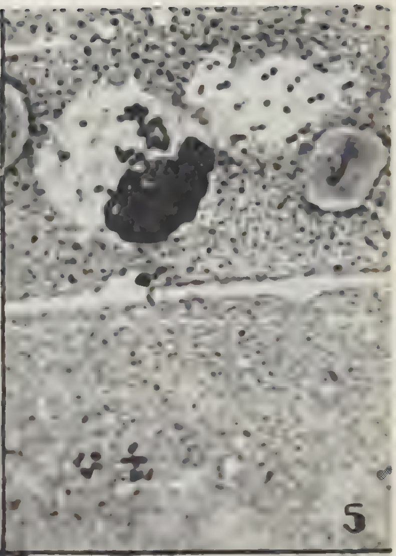


FIG. 1. Virgil Speed, Type VIII pneumonia, 5-6-38, 5 P.M., × 1700. Pre-therapy sputum specimen. Note the capsule halo and that each pneumococcus is isolated from others in the field.

FIG. 2. Virgil Speed, Type VIII pneumonia, 5-7-38, 12 midnight. × 1700. Sputum specimen taken 6 hours after the administration of 120,000 units of anti-pneumococcus horse serum. Note the clumping of extra-cellular capsulated pneumococci.

FIG. 3. Virgil Speed, Type VIII pneumonia, 5-7-38, 6 P.M., × 1700. Sputum specimen taken 3 hours after the administration of 120,000 units of anti-pneumococcus horse serum. Note the phagocytosis of capsulated pneumococci and the clumping of the extra-cellular organisms.





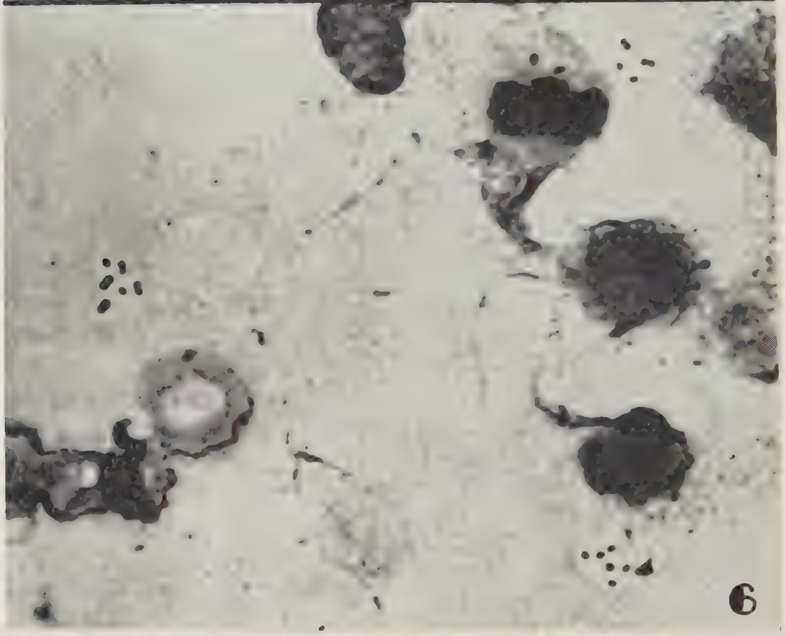


FIG. 4. Virgil Speed, Type VIII pneumonia, 5-8-38, 6 P.M., × 1700. Sputum specimen taken 24 hours after the administration of 120,000 units of anti-pneumococcus horse serum. Note that each phagocytized diplococcus stands out discreetly but that the capsules are not readily visualized. In addition, the individual extra-cellular pneumococci show less tendency to clump.

FIG. 5. Virgil Speed, Type VIII pneumonia, 5-7-38, 12 midnight, × 1700. Sputum specimen taken 6 hours after administration of 120,000 units of anti-pneumococcus horse serum. Note the clumps of uncapsulated diplococci within the cytoplasm of the phagocyte and also the extra-cellular clumps of both capsulated and uncapsulated organisms.

FIG. 6. David Alonzo, Type I pneumonia of 62 hours' duration. Pre-therapy sputum specimen. Note the clumps of extra-cellular capsulated pneumococci.

(Photographs by C. G. Eddy, Eloise Hospital, Eloise, Michigan.)

pneumococcic serum administration as evidenced in the sputum 3 to 24 hours after treatment with the arrangement of the extra-cellular capsulated organisms into definite small or large clumps suggestive of agglutination (Fig. 2). At the same time or shortly thereafter, it was often but not always possible to demonstrate an increase in the number of capsulated diplococci per phagocyte as illustrated in Figs. 3 and 4. The capsules of the extra-cellular pneumococci gradually decreased in size and these organisms also decreased in number as the sputum began to lose its rusty character. In most serum-treated cases, however, while the capsulated diplococci decreased, the total number of uncapsulated extra-cellular organisms remained high and in such specimens one obtained marked phagocytosis of clumps of these organisms as illustrated in Fig. 5.

Although clumping of capsulated pneumococci was usually associated with serum therapy, it has also been observed in the sputum of 4 patients who had not yet received treatment (Fig. 6). One of these patients entered the hospital with many Type II and a few Type IX pneumococci in the sputum. On the day of admission his serum agglutinated Type II pneumococcus to a titer of 1:40 but failed to react with Type IX. He recovered promptly without treatment. The phenomenon of clumping has also been seen rather frequently on the sixth to the tenth day of the pneumonia in patients who were under treatment with sulfanilamide but appeared to have no relation either to the dose or to the period of administration of the drug.

*Summary.* Three types of phagocytosis depending upon the degree of encapsulation of the engulfed pneumococci, have been observed in the sputum of patients suffering from lobar pneumonia. Clumping of capsulated pneumococci similar to agglutination and an increased phagocytosis of these organisms was evident in the sputum shortly after the administration of specific horse or rabbit antibody. Spontaneous clumping of capsulated pneumococci was observed in the sputum of 4 patients prior to therapy suggesting that a similar mechanism is involved in the development of active immunity. Further studies on the relation of the above phenomena to the clinical condition of the patient and the adequacy of serum dosage are now in progress.

### 10245

## Bactericidal Action of Pectin Containing Nickel.*

Edith Haynes, Charles A. Tompkins, Grace W. Crook and Matthew Winters. (Introduced by O. M. Helmer.)

*From the Central Laboratories and the Department of Pediatrics, Indiana University Medical Center, Indianapolis.*

In a preliminary report on the bactericidal action of pectin, evidence was presented to show that the pectin used,† hereinafter referred to as pectin "A", in a pH range of 5.0 to 5.5 and after being subjected to certain thermal conditions, had a bactericidal action on *E. coli.*[1] At that time it was not understood to what this action was due. The possibility of a chemical contaminant had been immediately considered but assurance had been given by the producers‡ that all pectin was very closely checked for sulphur. lead, arsenic and other metals and that no impurity was then known which could account for the bactericidal action.

Further work was done on the action of unautoclaved pectin "A" on *E. coli,* the effect of autoclaved pectin "A" on a number of other organisms, the action of pectins from other producers§ and the action of 2 derivatives of pectin, namely pectic acid and methyl *d*-galacturonate. After this work was completed we were informed by the producers‖ of pectin "A" that nickel was present in their product. The nickel is probably picked up during processing when the pectin solution is evaporated on a heated nickel drum. We have confirmed the presence of nickel in the pectin "A" and have tested the bactericidal activity of other samples of this pectin which were found to have varying amounts of nickel. The results of these tests, together with those of the work mentioned above, are presented here.

The method used for the bacterial counts was essentially the same as described in the first paper. *Escherichia coli* was again used and in addition *Staphylococcus aureus, Shigella paradysenteriæ* Flexner, *Pseudomonas aeruginosa, Erwinia caratovora,* a *beta* hemo-

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* Aided by a grant from Mead Johnson and Company, Evansville, Indiana.

† Generously furnished by Sardik Laboratories, Inc., New York City.

[1] Haynes, E., Tompkins, C. A., Washburn, G., and Winters, M., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 839.

‡ Communication from Sardik Laboratories, Inc., New York City, April, 1936.

§ Kindly furnished by General Foods Corporation, Battle Creek, Michigan, and California Fruit Growers Exchange, Ontario, California.

‖ Communication from Sardik Food Products Corporation, New York City, May, 1938.

lytic streptococcus and a diplostreptococcus from a case of ulcerative colitis. In the series in which the pectin was not subjected to heat, the pectin was added to the sterile broth, permitted to stand for 4 to 12 hours to insure thorough mixing and a fairly stable pH, necessary pH adjustments were then made and the rest of the procedure was carried out as given before. The nickel content¶ (dimethylgloxime method) as well as the percent ash were determined on all pectins used.

The results given in Table I, show that when a 2% solution of pectin was used in heart infusion broth, pectin "A" was the only pectin tested which manifested bactericidal action. This action varied with the percentage of nickel present, no action being observed in a sample which contained as low as 0.16%. All other pectins, which were found to contain no nickel, as well as the pectic acid and methyl *d*-galacturonate, not only did not have an inhibitory action but permitted an abundant growth. The samples of pectin "A" having the higher percentages of nickel were found to have a markedly inhibitory action on *S. aureus, S. paradysenteriæ* Flexner, *P. aeruginosa, E. caratovora* and the *beta* hemolytic streptococcus as well as *E. coli* but none on the diplostreptococcus. (Table II.) When not autoclaved, pectin "A" showed a slight inhibitory action which was somewhat variable but was never as marked as with the autoclaved solutions.

TABLE I.

Typical Results of the Effect of 2% Solutions of Various Pectic Substances on Growth of *Escherichia coli* in Heart-infusion Broth.

| Test solution | pH | Autoclaved or unautoclaved | Original count per cc | Count after 48 hours per cc | Nickel content % | Ash content % |
|---|---|---|---|---|---|---|
| Pectin "A" | 5.0 | A | $2 \times 10^5$ | 0 | 0.78 | 1.57 |
| ” "A" | 5.1 | U | $3 \times 10^5$ | $2 \times 10^6$ | 0.78 | 1.57 |
| ” "A" | 5.3 | A | $1 \times 10^5$ | 0 | 0.22 | 1.2 |
| ” "A" | 5.0 | A | $8 \times 10^4$ | $9 \times 10^8$ | 0.16 | 1.5 |
| ” "B" | 5.3 | U | $9 \times 10^4$ | $1 \times 10^9$ | 0 | 0.59 |
| ” "B" | 5.3 | A | $9 \times 10^4$ | $8 \times 10^7$ | 0 | 0.59 |
| ” "C" | 5.0 | U | $5 \times 10^4$ | $2 \times 10^9$ | 0 | 2.07 |
| ” "C" | 5.4 | A | $2 \times 10^5$ | $1 \times 10^9$ | 0 | 2.07 |
| ” "D" | 5.3 | A | $8 \times 10^4$ | $8 \times 10^8$ | 0 | 3.14 |
| ” "E" | 5.3 | A | $3 \times 10^5$ | $4 \times 10^6$ | 0 | 4.67 |
| ” "F" | 5.2 | A | $1 \times 10^5$ | $7 \times 10^7$ | 0 | 3.95* |
| Methyl *d*-galacturonate | 5.4 | U | $9 \times 10^4$ | $3 \times 10^9$ | 0 | — |
| ” ” | 6.1 | U | $1 \times 10^5$ | $2 \times 10^9$ | 0 | — |
| Pectic acid | 6.2 | A | $2 \times 10^5$ | $4 \times 10^8$ | 0 | — |
| ” ” | 6.4 | U | $2 \times 10^5$ | $2 \times 10^8$ | 0 | — |
| Broth control | 5.3 | | $2 \times 10^5$ | $2 \times 10^9$ | — | — |
| ” ” | 6.1 | | $7 \times 10^4$ | $2 \times 10^9$ | — | — |

*2.5% calcium present.

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¶ Grateful acknowledgment is due Dr. R. N. Harger for his generous assistance.

TABLE II.

Typical Results of Effect of 2% Pectin "A" on Growth of Various Organisms in Heart-infusion Broth.

| | pH | Test organisms | Autoclaved or unautoclaved | Original count per cc | Count after 48 hours per cc |
|---|---|---|---|---|---|
| Pectin | 5.4 | *E. coli* | U | 7 x $10^4$ | 2 x $10^6$ |
| Control | 5.4 | " | | 1 x $10^5$ | 1 x $10^9$ |
| Pectin | 5.4 | " | A | 7 x $10^4$ | 4 x 10 |
| Control | 5.4 | " | | 7 x $10^4$ | 7 x $10^7$ |
| Pectin | 5.4 | *S. aureus* | A | 3 x $10^5$ | 2 x $10^3$ |
| Control | 5.4 | " | | 3 x $10^5$ | 6 x $10^8$ |
| Pectin | 5.3 | *S. paradysenteriæ* Flexner | A | 2 x $10^4$ | 0 |
| Control | 5.3 | " " | | 4 x $10^4$ | 2 x $10^7$ |
| Pectin | 5.5 | Hemolytic streptococcus* | A | 3 x $10^4$ | 0 |
| Control | 5.5 | " " | | 3 x $10^4$ | 1 x $10^7$ |
| Pectin | 5.2 | *E. caratovora*† | A | 1 x $10^5$ | 2 x $10^4$ |
| Control | 5.2 | " | | 9 x $10^4$ | 2 x $10^7$ |
| Pectin | 5.2 | *P. aeruginosa* | A | 1 x $10^5$ | 2 x $10^4$ |
| Control | 5.2 | " | | 1 x $10^4$ | 6 x $10^7$ |
| Pectin | 5.4 | Diplostreptococcus | A | 1 x $10^5$ | 8 x $10^7$ |
| Control | 5.4 | " | | 2 x $10^5$ | 9 x $10^7$ |

*Final plates made at end of 24 hours' incubation.
†Tubes and plates incubated at room-temperature.

Since we could demonstrate bactericidal action only with pectin "A", which was found to be the only pectin tested containing nickel, and the action seemed to be in direct proportion to the amount of nickel present, it would seem that our reported bactericidal activity is a characteristic of this particular pectin containing nickel rather than of pectin *per se*. In what form the nickel is present, why its activity increases with heating, why the addition of sufficient NaOH to bring the pH to above 5.5 decreases the action and whether other metals in similar combination would have similar activity are matters for further investigation.

Preliminary work on the treatment of wounds with pectins containing no nickel indicates that though the bactericidal activity may be a slight contributing factor, there must be some other explanation for the total therapeutic value of pectin.

*Summary*. Pectin containing 0.22% or more nickel, after being subjected to certain thermal conditions and in a certain pH range, was found to have a slow bactericidal action when tested with *E. coli* and certain other bacteria. Other pectins which contained no nickel failed to demonstrate this activity.

### 10246

## Isolation and Determination of Riboflavin Produced by Tubercle Bacilli in Culture Media.

C. H. Boissevain, W. F. Drea and H. W. Schultz.

*From the Laboratory of the Colorado Foundation for Research in Tuberculosis, Colorado College, Colorado Springs.*

Warburg and Christian[1] isolated from yeast the yellow ferment of which riboflavin is the active part. Adler and Euler[2] found riboflavin in *Thermobacter helveticum*, *Bacterium pasteurianum* and *Clostridium butyricum*. Pett[3] found it present in yeast, *Bacterium aerogenes* and *B. subtilis,* but absent in Aspergillus, Penicillium, *B. proteus vulgaris, B. coli* and *Staphylococcus albus.*

Several strains of tubercle bacilli produce a green pigment on Sauton's medium. Kolle[4] states that this was first observed by Lange and Piscatore at the time of the tragedy in Lübeck caused by contamination of BCG vaccine with the "Kiel" strain of tubercle bacilli. This strain could be recognized by its abundant pigment production after being recovered from the organs of children who had died from the administration of the contaminated vaccine. Kraus and Koref[5] denied that the production of pigment is an exclusive peculiarity of the "Kiel" strain and state that all tubercle bacilli produce a greenish yellow pigment which does not show any specific absorption. de Grolier[6] also found that all tubercle bacilli form some pigment but observed that only 3 strains produce large quantities of pigment and impart an intense green fluorescence to the culture medium, namely the "Kiel" strain and 2 attenuated human strains, $R_1$ of Trudeau and "Nathan Raw".

We have studied the pigment production of one human strain, H37, one attenuated human strain $R_1$, one bovine strain NJ, and one attenuated bovine strain BCG. We could confirm de Grolier's observation that the $R_1$ strain produces far more pigment than any of the other strains. An examinations of the fluorescence spectrum shows one band in the region from 494m$\mu$ to 614m$\mu$, where the riboflavin band occurs and another band in the adjoining blue region. This latter band belongs to a different pigment which is difficult to separate from the riboflavin. The following method was finally successful:

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[1] Warburg and Christian, *Biochem. Z.*, 1933, **266**, 377.

[2] Adler and Euler, *Z. Physiol. Chem.*, 1934, **225**, 41.

[3] Pett, *Biochem. J.*, 1935, **29**, 937.

[4] Kolle, *Deutsche med. Wochensch.*, 1930, **58**, 304, 992.

[5] Kraus and Koref, *Z. f. Tuberkulose,* 1933, **67**, 42.

[6] de Grolier, *Compt. rend. d. l. Soc. d. Biol.*, 1933, **113**, 1506.

Ten liters of a 6-weeks-old culture of *Mycobacterium tuberculosis* $R_1$ on Sauton's medium are heated for 30 minutes to 90°C, brought to pH 1 with HCl and filtered to remove bacilli and precipitated protein. The riboflavin is then absorbed on Fuller's earth, which is separated from the liquid by centrifuging, washed free from HCl, and eluted with 200 cc of a mixture of 1 pyridine, 1 methanol and 4 water. After centrifuging, the pyridine and alcohol are removed by distillation under diminished pressure and the process of absorption and elution repeated twice more.[7] After the pyridine and alcohol have been removed for the last time, the solution is diluted to 100 cc with water and extracted twice with 20 cc portions of 88% phenol. The riboflavin goes into the phenol which is then removed by ether in the presence of water.[8] By repeating this process several times the riboflavin is obtained free from the blue fluorescing pigment. By repeated precipitation from water with acetone and recrystallization from water the pure riboflavin is obtained as orange crystals that melt and decompose at 280°C. The fluorescence spectrum produced by ultraviolet light in a watery solution is identical with that of pure synthetic riboflavin, and extends from approximately 494m$\mu$ to 614m$\mu$.

For the quantitative determination of riboflavin in culture media the media are deproteinized with trichloracetic acid and the riboflavin absorbed on Fuller's earth and eluted as described above. The Fuller's earth sometimes causes difficulties by forming a colloidal solution in the eluent. This difficulty can usually be overcome by adding methyl alcohol after part of the pyridine and methyl alcohol have been removed by distillation under diminished pressure. In some cases it may be necessary to add salt to precipitate the colloidal earth, which does not affect the fluorescence. After complete removal of the pyridine and alcohol the solution is brought to a volume of 10 cc with water, and to a pH of 7.0. This solution is then placed in a quartz cell in front of a spectrograph and the fluorescence excited by ultraviolet light. A narrow beam of the light of a carbon arc filtered through a Wratten filter No. 18A, at right angles with the spectrograph slit, is focused on the cell by a quartz lens. The intensity of the fluorescent band produced on the photographic plate is then compared with the intensity of bands produced on the same photographic plate by riboflavin solutions of known strength. It is necessary to determine the approximate concentration of the unknown in a preliminary experiment, so as to have the standard and the unknown of nearly the same concentration. The amount of fluorescent light excited depends both upon the concentration of the riboflavin and on the intensity of the exciting light. Since the riboflavin solution absorbs part of the exciting wavelengths, their intensity becomes less in high concentrations of riboflavin. By having standard and unknown at approximately the same concentration this source of error is avoided.

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[7] Kuhn, Gyorgy, Wagner, Jauregg, *Berichte*, 1933, **66**, 317, 576, 1034.

[8] Greene and Black, *J. Am. Chem. Soc.*, 1937, **59**, 1820.

The following concentrations of riboflavin were observed:

| Strain of tbc bacillus | Amount of riboflavin in culture medium, gamma per cc |
|---|---|
| $R_1$ | 2.86 |
| BCG | 1.07 |
| Bovine | 0.85 |
| Human H37 | 0.50 |

The bovine strain is the most virulent of the four and the BCG the least virulent, but both produce less riboflavin than the $R_1$ strain. de Grolier[6] observed greatest pigment production in 2 human strains of reduced virulence, but also in the Kiel strain which was unfortunately virulent. Apparently there exists no close relationship between virulence and pigment production.

*Summary.* Riboflavin was isolated from Sauton medium on which $R_1$ tubercle bacilli had grown and the amount of riboflavin produced by 4 different strains of tubercle bacilli was determined by a method of fluorescence spectrography described in the paper.

### 10247 P

## Comparison of Bacteriostatic Effects of Sulfanilamide and Sulfapyridine (2 Sulfanilyl Aminopyridine) on Bacteria in Broth Cultures.*

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Fleming[1] has recently reported that sulfapyridine—2(sulfanilyl aminopyridine)—in concentrations of from 1:256,000 to 1:8,000 inhibited the development of large inocula of type 23 pneumococci

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* This study was supported by a grant from the Chemical Foundation, Inc., New York City.

[1] Fleming, A., *Lancet*, 1938, **2**, 74.

TABLE I.
Bacteriostatic and Bactericidal Effects of Three Concentrations of Sulfanilamide and Sulfapyridine upon Various Bacteria in Broth.

| Organism | Inoculum orgs/cc | Growth—Colonies/1 cc.<br>20 Hr. | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Sulfanilamide, mg% | | | Sulfapyridine mg% | | | |
| | | 10 | 50 | 90 | 10 | 50 | 90 | Control |
| *Staph. aureus* | 22 | ++++<br>190 Mil. | ++++<br>1 Bil. | ++++<br>108 Mil. | ++++<br>190 Mil. | ++++<br>93 Mil. | ++++<br>132 Mil. | ++++<br>390 Mil. |
| Alpha Strept. | 9000 | —<br>19,200 | lost | —<br>0 | —<br>15,800 | —<br>0 | —<br>0 | +++<br>148 Mil. |
| Beta Strept. Gr. B | 36 | —<br>119,000 | —<br>78,000 | —<br>14,300 | —<br>64,000 | —<br>7,000 | —<br>2,400 | +++<br>200 Mil. |
| Beta Strept. Gr. C | 177 | — | — | —<br>Not plated | — | — | — | — |
| Beta Strept. Gr. D | 27 | ++++ | ++++ | ++++<br>380 Mil. | ++++ | ++++ | ++++<br>500 Mil. | ++++<br>490 Mil. |
| *E. coli* | 49 | ++++<br>850 Mil. | +++<br>141 Mil. | ±<br>9 Mil. | ±<br>8 Mil. | —<br>649,000 | —<br>25,000 | ++++<br>1.04 Bil. |
| *E. typhi* | 1 | ++<br>94 Mil. | —<br>160 | —<br>74 | —<br><2 | —<br>0 | —<br>0 | ++++<br>170 Mil. |
| 44 Hr. | | | | | | | | |
| *Staph. aureus* | 22 | Not tested | | | | | | |
| Alpha Strept. | 9000 | —<br><1000 | +<br>lost | —<br><10 | —<br><1000 | —<br><10 | —<br>0 | ++++ |
| Beta Strept. Gr. B. | 36 | ++++ | ++++ | ±<br>±1000 | ++ | —<br>208 | —<br>7 | ++++ |
| Beta Strept. Gr. C. | 177 | —<br><100 | —<br><10 | —<br><100 | —<br><100 | —<br><100 | —<br><10 | +++<br>125 Mil. |
| Beta Strept. Gr. D | 27 | Not tested | | | | | | |
| *E. coli* | 49 | ++++ | ++++ | ++++ | ++++<br>Not plated | ++++ | ++++ | ++++ |
| *E. typhi* | 1 | ++++ | ++++ | ++++ | ++++<br>Not plated | ++++ | ++++ | ++++ |

++++, etc. = Different degrees of turbidity. — = No apparent growth. Mil. = Million. Bil. = Billion. <1000 = less than 1000.

in deleucocyted human blood. In the presence of leucocytes a concentration of 1:32,000 sulfapyridine killed an inoculum of 100,000 pneumococci. The drug had a similar effect upon hemolytic streptococci. Bacteriostasis was obtained in the absence of leucocytes, while in their presence actual killing occurred. A comparison of the efficacy of sulfanilamide and sulfapyridine in whole blood showed that the latter was the more effective against hemolytic streptococci and type 15 pneumococci. Fleming used the slide cell technic which he described some years ago. We[2] have already shown that sulfapyridine is as effective a bacteriostatic agent as sulfanilamide against a strain of *beta* hemolytic streptococcus (C203), and is somewhat more effective against Types I, II and III pneumococci in broth cultures. The present investigation was undertaken with the purpose of comparing the action of sulfapyridine and sulfanilamide against a miscellaneous group of microörganisms.

One strain each of *beta* hemolytic streptococci, Groups B, C, and D, *alpha* hemolytic streptococcus, *Staphylococcus aureus, E. coli* and *E. typhi* were tested for susceptibility to 10, 50, and 90 mg % concentrations of sulfanilamide and sulfapyridine in beef infusion broth (2% Neopeptone, 0.075% dextrose). A series of tubes, containing the desired concentrations of the 2 drugs in broth and a drug-free control, were inoculated with 0.5 cc of culture dilution. The cultures had been grown in broth at 37°C for 5 or 6 hours, and the dilutions, made with the same medium, were sufficiently high in most instances to give inocula of less than 100 organisms. The size of the inoculum and the extent of growth in the tests after 20 hours' incubation were determined by pouring plates and counting the colonies which developed. The tests were also examined visually for turbidity and those which showed less than normal growth were returned to the incubator and plates poured again at 44 hours. The results are shown in Table I.

Sulfapyridine was a somewhat more effective bacteriostatic agent than sulfanilamide in broth cultures of *E. coli, E. typhii* and Group B *beta* hemolytic streptococci. The two compounds were approximately equally active against Group C *beta* hemolytic streptococci and *alpha* streptococci, and they were equally inactive against Group D *beta* hemolytic streptococci and *Staphylococcus aureus.*

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[2] Long, P. H., Bliss, E. A., and Feinstone, W. F., *Pennsylvania Medical J.*, in press.

### 10248

# Observations upon the Absorption and Excretion of Sulfapyridine* (2 Sulfanilyl Aminopyridine).

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We[1] have already discussed the concentrations of sulfapyridine noted in the blood of mice, dogs and human beings following the administration of single doses of this drug. In this communication we wish to submit additional data concerning the absorption, distribution and excretion of sulfapyridine. In all of our experiments we have used Marshall's method[2, 3] and have found it highly satisfactory for the determination of sulfapyridine in body fluids.

In Table I are recorded the concentrations of the drug obtained in the blood of mice, dogs and human beings following single peroral doses. It is to be noted that the concentrations of sulfapyridine observed in these experiments are definitely lower in each species than those which have been reported for corresponding doses of sulfanilamide. It is also of interest that in the same dog, marked variations in the absorption of sulfapyridine were noted in experiments carried on at different times. In human beings also, a definite variation in the ability of individuals to absorb this compound was observed.

The absorption rate of sulfapyridine would seem to be slower than that of sulfanilamide, and because of this factor, the peak concentration of the drug observed in the blood of human beings occurs at somewhat later periods than have been noted for sulfanilamide. Because of this, we have concluded that in human beings the dosage of this compound should be based on a 6-hour schedule rather than upon a 4-hour schedule, as is the case in sulfanilamide therapy.

In Table II we have recorded observations of the concentrations of sulfapyridine in the blood of patients who were being regularly treated with this drug at 6-hour intervals. The bloods were obtained between 9 and 10 A.M.

It would seem from the data in this table, that regardless of size

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* This investigation was supported by a grant from the Chemical Foundation, Inc., New York City.

[1] Long, P. H., Bliss, E. A., and Feinstone, W. H., *Pennsylvania Med. J.*, in press.

[2] Marshall, E. K., Jr., *J. Biol. Chem.*, 1937, **122**, 263.

[3] Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 85.

TABLE I.
Blood Levels of Sulfapyridine Following Single Peroral Doses in Mice, Dogs and Man.

| Species | Dose, g/kilo | Blood levels in mg%, hours following administration of sulfapyridine | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 4 | | 5 | | 8 | | 24 | | 36 | | 48 | |
| | | F | T | F | T | F | T | F | T | F | T | F | T | F | T | F | T |
| Mouse | 0.5 | 15.4 | | 15.6 | | 12.5 | | | | 6.9 | | 3.2 | | | | | |
| ,, | 1.0 | 23.6 | 22.9 | | | 21.1 | 21.4 | | | 16.0 | 16.5 | 0.7 | 0.6 | | | 0 | 0 |
| Dog I | 1.0 | 0.8 | 0.8 | | | 6.25 | 6.25 | | | 9.1 | 9.1 | 1.0 | 1.0 | | | | |
| ,, | 1.0 | | | | | 19.4 | 19.5 | | | 16.7 | 16.8 | 1.3 | 1.4 | | | | |
| ,, | 1.0 | T | T | | | 9.35 | 9.44 | | | 17.1 | 17.3 | 1.2 | 1.3 | | | | |
| Man | 0.05 | 0.55 | 0.53 | | | 3.26 | 3.2 | | | 2.77 | 2.6 | 1.8 | 1.7 | 1.1 | 1.0 | | |
| ,, | 0.05 | T | T | | | 5.0 | 4.9 | | | 4.1 | 4.4 | 2.5 | | 1.6 | | | |
| ,, | 0.05 | 0.5 | 0.6 | | | 4.2 | 4.0 | | | 3.0 | 3.4 | 3.1 | 3.1 | 1.9 | 1.9 | | |
| ,, | 0.05 | 1.6 | 1.7 | | | 3.4 | 3.4 | | | 3.1 | 3.2 | 3.1 | 3.1 | | | 1.3 | 1.3 |
| ,, | 0.10 | 0.9 | 1.1 | | | | | 4.6 | 6.4 | 4.7 | 7.4 | 1.9 | 2.9 | 0.9 | 2.4 | 0.4 | 0.7 |
| Infant | 0.1 | | | 3.2 | 3.2 | 5.6 | 5.6 | | | 5.3 | 5.4 | 2.4 | 2.5 | | | VFT | VFT |
| ,, | 0.1 | | | 4.1 | 4.1 | 3.3 | 3.6 | | | 3.2 | 3.5 | 2.0 | 2.3 | | | VFT | VFT |

T = Total sulfapyridine (including conjugated fraction).

TABLE II.
Concentrations of Sulfapyridine Noted in Blood of Patients Receiving This Compound.

| | | | Blood Levels mg%—Day of Administration of Drug | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | |
| Subject | Wt, kilo | Dose per day, g | F | T | F | T | F | T | F | T | F | T | F | T | F | T | F | T |
| G.B. | 62 | 4 | | | 1.7 | | | | 7.1 | 7.1 | | | | | 6.3 | 7.1 | | |
| S. D. | — | 6 | 1.6 | 1.8 | 3.1 | 4 | 2 | 3.8 | 2.8 | 4.6 | 1.5 | 3.2 | 1.8 | 3.9 | | | 1.9 | 3.8 |
| N.M. | 38 | 6.9 + 2 | 6.2 | 6.3 | 3.1 | 3.5 | 3.8 | 3.8 | | | 1.1 | 1.1 | 0.8 | 0.8 | | | | |
| J.B. | 60 | 3.6 | 2.6 | 3.5 | | | | | | | | | | | | | | |
| J.R. | 60 | 3.6 | | | | | 6.5 | 6.5 | | | | | 9.0 | 9.5 | | | | |
| L.A. | 42 | 3.6 | | | T | 1.0 | | | 3.0 | 4.2 | | | 3.7 | | | | 2.4 | 3.2 |
| D.C. | 49 | 3.6 | 1.1 | | | | | | 2.0 | | | | 2.1 | | | | 1.7 | 2.2 |
| F.P. | 71 | 4.8 | 7.7 | | | | 6.5 | | | | | | 3.0 | | | | 4.9 | |
| B.F. | 63 | 3.6 | 6.7 | 6.7 | | | 4.5 | 5.0 | | | | | 5.3 | 5.2 | | | | |
| G.J. | 70 | 12.0 | 4.8 | 8.6 | 2.8 | 6.6 | 3.8 | 6.2 | 4.0 | 6.8 | | | 2.3 | 5.7 | 3.8 | 9.6 | 4.0 | 10.0 |

F—Free sulfapyridine.
T—Total sulfapyridine (including conjugated fraction).

TABLE III.

Urinary Excretion of Sulfapyridine Following Administration of Single and Repeated Doses of the Compound.

| | | Volume of Urine, Excretion in mg%, Days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dose of | 1 | | | 2 | | | 3 | | | 4 | | | Total | |
| Subject | compound | U.V. cc | F | T | U.V. cc | F | T | U.V cc | F | T | U.E. cc | F | T | drug excreted, g | % of dose excreted |
| J.C. | Single<br>0.05 g/kilo<br>Total = 3.0 | 1875 | 32.6 | 48.6 | 2775 | 23.5 | 36.6 | 1875 | 11.6 | 25 | 1660 | 6.4 | 13.2 | 2.37 | 79 |
| S.B. | Single<br>0.1 g/kilo<br>Total = 5.7 | 2850 | 25 | 48.8 | 2600 | 11.9 | 45.5 | 1650 | 4 | 22.5 | | T | T | 2.9 | 51 |
| E.P. | Single<br>0.1 g/kilo<br>Total = 6.8 | 1675 | 50 | 79 | 900 | 31.2 | 141.5 | 1000 | 4 | 16.8 | | T | T | 2.34 | 39 |
| J.B. | 3.6 g<br>per day | 1440 | 30 | 155 | 1520 | 40.8 | 137 | 1350 | 53.6 | 166 | | | | 6.55 | 60 |
| H.W. | 3.6 g<br>per day | 1840 | 62.5 | 105 | 1780 | 47 | 89.9 | 2640 | 40 | 77 | | | | 5.52 | 51 |
| D.J. | 3.6 g<br>per day | 2130 | 36.4 | 107 | 2240 | 31.3 | 95.7 | 2310 | 38.1 | 111 | | | | 6.97 | 64 |

U.V. = Urine volume.
F = Free sulfapyridine.
T = Total sulfapyridine (includes conjugated fraction).

of the dose of the drug, marked variations occur in the concentrations observed in bloods from various patients. These findings are in contrast to the rapid and nearly complete absorption of sulfanilamide observed in human beings. These marked irregularities in the absorption of the drug necessarily make accurate therapy with sulfapyridine difficult.

Marshall and his associates[2] first observed that sulfanilamide was almost totally excreted in the urine of individuals possessing normal renal function. This has been confirmed by Stewart, *et al.*[4] In Table III are presented experiments dealing with the urinary excretion of sulfapyridine.

It seems quite definite that, following the administration of a single dose of sulfapyridine, its excretion in the urine is not complete until 3 or 4 days have passed and that the amount of the drug excreted varies markedly in normal individuals. If, on the other hand, repeated doses of the compound are given to patients at 6-hour intervals over a period of days, and estimations be made of the total amount of the drug excreted over a 3-day period, it will be seen that from 50 to 65% of the ingested sulfapyridine has been excreted. This is a decidedly lower figure than was obtained with sulfanilamide, and suggests that large doses of sulfapyridine are not necessarily indicated because much of the drug may not be absorbed.

It has been observed[2] that from 10 to 20% of circulating sulfanilamide exists in a conjugated form (acetyl sulfanilamide) in the blood of patients treated with this drug. Our studies tend to indicate that the percentage of conjugated sulfapyridine in the circulating blood of patients treated with sulfapyridine, may reach a high figure and that, in general, relatively more of the drug is conjugated than is the case when sulfanilamide is administered to human beings. This is again a detriment to therapy because conjugated sulfapyridine probably has little chemotherapeutic activity.

We have observed that sulfapyridine passes over into the spinal fluid of human beings in from one-half to three-quarters of the concentration of the drug which exists in the blood. It is present in purulent pleural exudates in concentrations of about 75% of that which exist in the blood. Hence, it seems that the drug diffuses into the tissues somewhat less readily than does sulfanilamide.

*Conclusions.* Sulfapyridine (2-sulfanilyl aminopyridine) is less readily and more irregularly absorbed by human beings than is sulfanilamide. Our experience leads us to believe that from a half to two-thirds of the ingested drug is excreted in the urine. Sulfapyridine is found in purulent pleural exudates and spinal fluids in concentrations of a half to two-thirds of those observed in the blood. In the blood of human beings a considerable fraction of the drug is frequently found in the conjugated form. Because of the irregular absorption of the drug and its tendency towards conjugation, accurate therapy with sulfapyridine is more difficult than with sulfanilamide.

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[4] Stewart, J. D., Rourke, G. M., and Allen, J. G., *J. A. M. A.*, 1938, **110**, 1885.

### 10249

## Note on the Mechanism of Specific Agglutination.

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One consequence of the "lattice"-theory of serological reactions* is that if *2* or more independent antigens and their respective antibodies are all mixed together, each system should aggregate independently. By using microscopically visible and distinguishable particulate antigens† (*e.g.,* bacteria and/or erythrocytes) this prediction has been recently tested.[1-5] In many cases the individual aggregates were chiefly or entirely composed of only one of the 2 kinds of antigenic particles, but the observation has also been made, which seems difficult to account for on the basis of the lattice-theory, that in some cases the aggregates are heterogeneous, *i.e.,* contain both kinds of antigenic particles. Mixed aggregation is also indirectly indicated by the results of experiments with precipitins,[3, 4] where the rate of flocculation in mixed systems is definitely accelerated in the zones of equivalence and excessive antibody where the increased cohesive effect due to combined antibody (plus electrolyte and lipin) could be expected to exert a dominant influence.

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* "This view differs from the hypothesis that the antibody globulin is denatured in that the aggregation of the particles of antibody combined with antigen is ascribed not only to a loss of attraction for water, but also to a *specific* attraction between the particles. *This mutual attraction is due to the link provided by further antigen molecules.*" (Italics ours.) ". . . . the formation of a continuous lattice fails progressively more and more as no further antigen molecules are available to provide links for the formation of larger structures when aggregates of larger size . . . . . are formed." (Marrack[6]).

Heidelberger and associates similarly picture the precipitate[7] (or agglutinate[8]) as a lattice-structure *in which antibody and antigen alternate.*

† Landsteiner reported similar experiments long ago (*Hand. Biochem.,* 1909, **2**, 400).

[1] Topley, W. W. C., Wilson, J., and Duncan, J. T., *Brit. J. Exp. Path.,* 1935, **16**, 116.

[2] Abramson, H. A., *Nature,* 1935, **135**, 995.

[3] Hooker, S. B., and Boyd, W. C., *J. Immunol.,* 1937, **33**, 337.

[4] Duncan, J. T., *Brit. J. Exp. Path.,* 1938, **19**, 328.

[5] Wiener, A. S., in press.

[6] Marrack, J. R., *The Chemistry of Antigens and Antibodies,* London, 1938.

[7] Heidelberger, M., and Kendall, F. E., *J. Exp. Med.,* 1935, **61**, 563; **62**, 467.

[8] Heidelberger, M., and Kabat, E. A., *J. Exp. Med.,* 1937, **65**, 885.

It should be pointed out that the occurrence of homogeneous aggregates, though compatible with the lattice-theory, does not actually prove that the lattice-mechanism was in fact operative in building up the aggregates. Antibody-molecules are invisible and the postulated alternation of antibody and antigen-particles cannot actually be observed. The existing evidence would support equally any hypothesis calling for a certain amount of specificity in the second stage. Also the supposed analogy with crystal-formation is imperfect because for the lattice-structure of some crystals it is not required that *different* kinds of atoms be alternately interposed.

The essential difference between the lattice-hypothesis and the older concept concerns the mechanism by which the primary compounds of antigen and antibody combine to form aggregates (agglutinated cells or particles of precipitate). The older concept pictured the primary compound as having a nucleus (molecule or particle) of multivalent antigen more or less enveloped by molecules of antibody-globulin; such a coated particle combines with another or with an aggregate of coated particles, because of some attraction or perhaps sometimes a simple lack of repulsion between the coated surfaces. The completeness of this coating would presumably depend on the number of reactive sites (valency) of the particles; with insufficient antibody, all of these sites would not be covered. This attractive force has commonly been thought to be non-specific in the immunochemical sense.

In addition to considerations previously reviewed, this nonspecificity is also indicated by certain observations recently called to our attention by Landsteiner: precipitates (cf.[9]) and agglutinates sometimes exhibit a tendency to stick to the walls of the test-tube, and in the case of cells agglutinated by immune chicken serum, as we ourselves have seen, this tendency is sometimes very powerful, so that the agglutinated mass literally has to be scraped from the glass.

However, the well confirmed observation of homogeneous aggregates in mixed systems quite properly suggests that some sort of specific mechanism be considered, although the observation of heterogeneous aggregates likewise suggests that the specificity is either not of a high order or not always operative.

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[9] Hooker, S. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 911.

The newer theory pictures a particle of antigen, combined with a molecule of antibody, as now either combining through the antigenic part with another molecule of antibody, or, through the antibody-part, with another particle of antigen. Or, in either case, with a particle of antibody or antigen, respectively, already forming part of an aggregate, but having a reactive area still available. Both antibody and antigen must be assumed to be multivalent. A particle of antigen completely coated (at all available reactive areas) with antibody could only combine with a free particle of free antigen or an uncombined reactive site on a particle of antigen already in combination. Two particles of antigen, both so completely coated that no reactive groups are exposed, *should not be able to combine with each other* according to the theoretical lattice-mechanism " . . . the formation of a continuous lattice fails . . . . as no further antigen molecules are available to provide links . . . . "

This prediction of the lattice-theory can readily be tested by direct observation if we use antigenic particles visible under the microscope. We have been led by such considerations to some extremely simple experiments whose results give *no indication that lattice-formation plays any part whatsoever in the mechanism of the following typical instance of serological agglutination.*

A drop of very dilute erythrocyte-suspension was added to 3 drops of strong hemagglutinin (immune rabbit serum diluted 1:10). After centrifugation, the supernate of course still powerfully agglutinated fresh cells so it is certain that an excess of antibody was present. Nevertheless, more agglutinin was added to the original agglutinate although this made no difference in the outcome of the experiment. The tube was shaken after the addition of fresh agglutinin; the clump did not break up, but floated as one homogeneous mass in the fluid. After half an hour this clump and several others similarly prepared were placed in the same tube and centrifuged. The result was the formation of one firm clump which did not break up, even on violent shaking.

Inasmuch as at least 10,000-fold excess of serum was used there can be no doubt that every cell in these original treated aggregates, and in particular every cell on the surface, was maximally coated with antibody. According to the lattice-theory, such clumps should have not been able to combine; nevertheless they did so.

Even if the aggregates after being placed together were not centrifuged, but simply allowed to settle, they usually united, in some cases so firmly that they could not be separated by any shaking less violent than that necessary to break up the original aggregates themselves. Thus the use of the centrifuge does not alter this experiment in any essential way, but simply accelerates the process and brings the aggregates in better contact.

It might be thought that the behavior of these relatively large aggregates would possibly be different from that of single coated antigenic particles or small aggregates. That this is not so is shown by the following experiment: a drop of very dilute suspension was mixed with a drop of strong agglutinin, and immediately placed under a large cover slip on a slide. If this preparation were not moved or shaken, microscopic observation even after half an hour revealed many small clumps of 2, 3, or a few cells, and many individual cells. This was clearly due to the poor mixing under these conditions, which prevented the encounters between coated cells and small clumps necessary for the formation of larger clumps; a similar preparation, when well mixed, agglutinated almost instantly into a few large clumps. There can hardly be any doubt, however, that the cells were one and all completely coated with antibody on all their reactive sites, for antibody was present in great excess, and the primary reaction is known to be extremely rapid. For instance, such mixtures, or others containing 1/100 as much antibody, can be centrifuged immediately after mixing, with no resultant weakening of agglutination which might indicate that sufficient antibody had not already been taken up. Also since it may be calculated (cf.[10]) that antibody-molecules at room-temperature have velocities of the order of half a meter per second, due to Brownian motion, it can be seen that in the time elapsed there must have been amply sufficient impacts between antibody-molecules and erythrocytes to coat the latter to a maximal degree, as is in any case deducible from general experience with agglutinative reactions. Also it was evident from the appearance of the clumps as the preparation stood on the slide that though they were composed of only a few, or in some cases 2, cells, nevertheless the cells were coated with an enormous excess of agglutinin, for the cells, typically, became greatly distorted, and tended to coalesce, though remaining unruptured.

When after the lapse of half an hour the contents of such a drop were thoroughly mixed, the small clumps and single cells immediately united to form 2 or 3 large clumps, and microscopic observation of the fluid showed that it was entirely clear, with no single cells or small clumps remaining. According to the lattice-theory, the coated single cells should not have been able to combine with anything except uncoated cell-surfaces, which were not present; but actually they did combine, obviously either with other coated single cells or with small clumps of coated cells.

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[10] Taylor, H. S., *A Treatise on Physical Chemistry*, N. Y., 1924, p. 1279.

These experiments clearly show that the lattice-mechanism, in the sense of aggregation through links of antigen, is not involved in agglutination, either in the later or the earlier stages under the conditions described. In fact it does not seem too much to say that if the lattice-theory were correct, then agglutination, as it is actually often observed to take place, would be impossible. There were simply no particles of antigen to serve as links, but nevertheless agglutination promptly and normally occurred. It is evident from kinetic considerations that the primary process of coating the cells would, considering the enormous disparity between the (Brownian) velocities of protein-molecules and of cells, be complete long before any large number of encounters between cells had taken place.

Similar microscopical observations cannot be made in the case of molecularly dispersed antigens. The macroscopical observation that small aggregates, even after long contact with excessive antibody, will unite, can, however, be made, and seems to us almost equally conclusive. Also, as we previously pointed out, since it is routinely observed that no excess of *antibody,* no matter how great, will prevent precipitation, if sufficient antigen to give a visible precipitate under any conditions is present, it may be concluded that the presence of molecules of antigen to serve as links is not necessary for the progress of the precipitin-reaction. For in the mixture containing the larger amounts of antibody it seems almost necessary to suppose that the primary reaction would have progressed practically to completion‡ before the secondary aggregates had reached any considerable size, and consequently, if the lattice-theory were true, flocculation would cease at this point, due to a lack of molecules of antigen to serve as links. Actually it is found that in the case of mixtures containing sufficient (2 or 3 times optimal) amounts of antibody, decrease in the amount of antigen used simply results in a proportional increase in the time that elapses before visible particles appear. In such mixtures a linear relation was found[11] between the dilution of the antigen and the time of flocculation. We concluded that the relative or perhaps absolute deficiency of molecules of antigen to act as links, which must have soon existed in the mixtures containing the more dilute antigen, did not exercise any influence on the flocculation, but instead the slowing of aggregation was adequately explained, to use Marrack's own words, by " . . . the reduction in the number of antigen molecules which serve as centers of aggregation."

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‡ This would seem to be a valid deduction from kinetic theory, allowing for the great excess of one reagent (antibody), and the greater velocities of the uncombined molecules (see above).

[11] Hooker, S. B., and Boyd, W. C., *J. Gen. Physiol.*, 1935, **19**, 373.

Experiments similar to those outlined above can be carried out when antibody is not in great excess, but it is then harder to be quite certain that the antigenic particles are completely coated, and this is vital to the argument. We have, however, previously found that "in mixed preparations containing much less than the optimal amount of antibody . . . . there seemed to be a tendency for one or the other kind of cell to preponderate" (in the aggregates). We have also noted that when independent precipitin-systems, containing an excess of antigen, are mixed, acceleration is either less than expected or lacking. It is perhaps not inconceivable that in mixtures where antigen is in excess the lattice-mechanism, or something like it, may be partly operative, though we have as yet no convincing proof that this is the case.

The older theory may have to be altered to allow for a certain order of specificity in the stage of secondary aggregation. However, it is by no means yet proved that this specificity is at all comparable in kind or degree with the specificity of primary immunochemical combination. In any case, the older theory will be here under no disadvantage compared with the lattice-theory, which explains this specificity only by postulating a mechanism that can be shown experimentally not to be operative in the sense that particles or small aggregates of antigen, fully coated with antibody insofar as the antigenic structure of their surface permits them ever to be coated, can and do combine with each other without the intermediation of fresh particles of antigen to serve as links.

The authors are indebted to L. G. Boyd for helpful suggestions.

### 10250

## Blood Volume, Cardiac Size and Renal Function in Dogs with Hypertension Produced by Goldblatt Technic.

John G. Gibson, 2nd, and Roger W. Robinson. (Introduced by Henry A. Christian.)

*From the Department of Medicine, Harvard Medical School, Boston.*

Freeman and Page[1] found no significant changes in plasma volume during a period of hypertension experimentally produced by the Goldblatt technic,[2] in 3 dogs, 2 of which were completely sympathectomized. We have followed the changes in plasma, cell and total volume by the dye method of Gibson and Evans[3, 4] in 5 female dogs, none of which were sympathectomized, during periods of hypertension produced by the Goldblatt technic, varying from 1 to 10 months, in an attempt to correlate observed changes in blood volume with changes in mean arterial pressure, renal function and cardiac size as determined by X-ray.

Control mean arterial pressure was established by an average of 12 determinations by femoral puncture over a period of 1 month prior to operation. In the operative procedure the renal artery clamp was completely closed and then released 1 to 1½ turns. Constriction of the right renal artery was effected in each animal first, followed by constriction of the left renal artery at a subsequent operation 2 weeks later. Recovery from the surgical procedure was uneventful in all except Dog 23 in which, following the constriction of the left renal artery uremia developed. Following the complete release of the clamp on the left renal artery this animal survived but a chronic azotemia persisted.

Renal function was estimated by determinations of phenolsulphonephthalein excretion and blood urea nitrogen.

Results are summarized in Table I.

Control mean arterial blood pressures were normal in all dogs. Following constriction of the right renal artery, there was no change in pressure in 1 dog, a transient rise in 1 dog and sustained increase of from 30 to 50 mm Hg in 3 dogs.

In the 4 dogs in which both renal arteries were constricted, pres-

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[1] Freeman, N. E., and Page, I. H., *Am. Heart J.*, 1937, **14**, 405.

[2] Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W., *J. Exp. Med.*, 1934, **59**, 347.

[3] Gibson, J. G., 2d, and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 301.

[4] Gibson, J. G., 2d, Keeley, J. L., and Pijoan, M., *Am. J. Physiol.*, 1938, **121**, 800.

TABLE I.

Plasma, Cell and Total Blood Volume, Mean Arterial Tension and Renal Function in Dogs Rendered Hypertensive by the Goldblatt Technic.

| | | Blood Volume | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Plasma | | Cell | | Total | | | | | | |
| Date | Wt, kg | cc | cc per kg | cc | cc per kg | cc | cc per kg | Hematocrit | Mean arterial blood pressure, mm Hg | P.S.P. % | B.U.N. mg % | Remarks |
| | | | | | | | Dog No. 23. | | | | | |
| 3/25/37 | 17.4 | 998 | 57.2 | 657 | 37.7 | 1655 | 95.1 | 39.9 | 110 | 60 | 16 | |
| 4/13 | | | | | | | | | | | | Right renal artery clamped |
| 4/22 | | | | | | | | | 140 | | | |
| 5/4 | | | | | | | | | 120 | | | Left " " " |
| 9/27 | 16.4 | 1105 | 67.3 | 1080 | 65.8 | 2185 | 133.1 | 49.4 | 220 | 65 | 60 | |
| 10/19 | 17.2 | 1268 | 73.7 | 1127 | 65.5 | 2395 | 139.3 | 47.03 | 185 | 60 | 60 | |
| 11/30 | 18.0 | 947 | 52.6 | 928 | 51.5 | 1875 | 104.3 | 49.6 | 195 | | | |
| | | | | | | | Dog No. 34. | | | | | |
| 7/27 | 15.3 | 850 | 55.5 | 695 | 45.4 | 1545 | 101.0 | 45.9 | 120 | 85 | 16 | |
| 8/23 | | | | | | | | | | | | Right " " " |
| 8/30 | | | | | | | | | 110 | | | |
| 9/2 | | | | | | | | | 110 | | | Left " " " |
| 10/13 | 14.4 | 1063 | 73.9 | 722 | 50.1 | 1785 | 124.0 | 40.4 | 165 | 85 | 15 | |
| 11/30 | 13.1 | 772 | 58.7 | 848 | 64.7 | 1620 | 123.5 | 52.4 | 170 | 80 | 18 | |
| 1/12 | 15.2 | 986 | 64.8 | 829 | 54.5 | 1815 | 119.4 | 45.7 | 165 | 80 | 16 | |
| 5/12 | 18.0 | 1120 | 62.2 | 1130 | 62.2 | 2250 | 125.0 | 50.3 | 150 | 75 | 14 | |
| | | | | | | | Dog No. 35. | | | | | |
| 7/27 | 20.0 | 1005 | 50.3 | 1530 | 76.5 | 2535 | 126.8 | 60.2 | 120 | 70 | 16 | |
| 8/26 | | | | | | | | | | | | Right " " " |
| 9/2 | | | | | | | | | 140 | | | |
| 9/6 | | | | | | | | | 150 | | | Left " " " |
| 9/10 | 21.8 | 1100 | 50.5 | 1440 | 66.0 | 2550 | 117.0 | 56.8 | 205 | 70 | 26 | |
| 10/18 | 19.6 | 1070 | 54.7 | 1470 | 75.0 | 2540 | 129.5 | 57.7 | 225 | 75 | 21 | |
| 11/15 | 21.0 | 1093 | 52.0 | 1277 | 60.8 | 2370 | 113.0 | 54.0 | 220 | 75 | 15 | |
| 11/23 | 21.3 | 1025 | 48.2 | 1065 | 50.0 | 2090 | 98.0 | 50.8 | 200 | 75 | — | |
| 1/11 | 23.4 | 953 | 40.7 | 1297 | 55.9 | 2250 | 96.2 | 57.6 | 205 | 70 | 18 | |
| 5/12 | 23.2 | 1320 | 56.9 | 1040 | 44.8 | 2360 | 101.5 | 44.0 | 185 | 70 | 15 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Dog No. 37. | | | | | |
| 9/27 | 13.2 | 945 | 71.5 | 575 | 43.5 | 1520 | 115.2 | 37.9 | 110 | 65 | 15 | |
| 9/28 | | | | | | | | | | | | |
| 10/1 | | | | | | | | | | | | Right “ “ “ |
| 10/4 | | | | | | | | | 145 | | | |
| 11/8 | | | | | | | | | 160 | | | Left “ “ “ |
| 12/6 | 14.9 | 715 | 48.0 | 615 | 47.9 | 1330 | 89.3 | 46.1 | 215 | 80 | 21 | |
| 1/11 | 14.1 | 883 | 62.6 | 932 | 66.1 | 1815 | 128.5 | 51.3 | 190 | 80 | 18 | |
| 5/12 | 16.2 | 796 | 49.1 | 939 | 57.9 | 1735 | 107.1 | 54.1 | 195 | 80 | 13 | |
| | 16.6 | 749 | 45.0 | 846 | 51.0 | 1595 | 96.3 | 53.1 | 185 | 60 | 19 | |
| | | | | | | | Dog No. 39. | | | | | |
| 10/19 | 14.0 | 856 | 61.2 | 639 | 45.7 | 1495 | 106.8 | 42.8 | 100 | 65 | 14 | |
| 10/21 | | | | | | | | | | | | Right “ “ “ |
| 10/29 | 14.4 | 733 | 50.8 | 747 | 51.8 | 1480 | 102.5 | 50.4 | 170 | 60 | 16 | |
| 11/8 | 14.4 | 662 | 45.8 | 653 | 45.2 | 1315 | 91.4 | 49.7 | 155 | — | — | |
| 11/15 | 14.6 | 622 | 42.5 | 716 | 49.0 | 1338 | 91.8 | 53.5 | 175 | — | — | |
| 11/24 | | | | | | | | | | | | Right nephrectomy |
| 12/7 | 16.4 | 760 | 46.2 | 685 | 41.7 | 1445 | 88.2 | 47.5 | 115 | 40 | 24 | |
| 12/20 | 15.0 | 700 | 46.7 | 605 | 40.3 | 1305 | 87.1 | 46.3 | 105 | 80 | 13 | |
| 1/3 | 15.5 | 807 | 52.0 | 643 | 41.4 | 1405 | 93.6 | 44.4 | 120 | 60 | 18 | |

sure rose on an average of 85 mm Hg above the control level. There was a gradual decline over a 3 to 6 months' period to levels of about 175 mm Hg, which were thereafter sustained throughout the course of the observation period. In Dog 39, following constriction of the right renal artery only, pressure rose from 110 to 170 mm Hg, ranged between 155 to 175 mm Hg for a month and following right nephrectomy fell promptly to the control level; and remained within normal limits thereafter.

Absolute plasma volume fluctuated throughout the period of observation, increases ranging from 160 to 270 cc and decreases of from 50 to 230 cc. In 4 dogs the total volume of red cells increased in 3 and decreased in 1, as did total blood volume. While these fluctuations were considerable, they bore no apparent relationship to the degree or duration of elevation of mean arterial pressure. In Dog 39, in which the period of pressure elevation was accompanied by a decrease in plasma and total volume, removal of the ischemic kidney produced a prompt return of mean arterial pressure to normal, and the blood volume rose to the control level simultaneously.

There was definite cardiac hypertrophy as shown by X-ray in the 4 dogs undergoing bilateral constriction of the renal arteries, the transverse diameter having been increased on an average of 1.4 cm and the diagonal measurement by 1.6 cm. None of these animals showed any signs or symptoms of congestive heart failure.

Renal function was unimpaired, both blood urea nitrogen and phenolsulphonephthalein excretion being within normal limits throughout the period of observation in 4 dogs. In Dog 23, in which uremia and subsequent azotemia developed, the blood urea nitrogen remained elevated and phenolsulphonephthalein excretion diminished.

During the period of hypertension no constant trend in the course of the blood volume was observed. While the volume was increased in some animals, the finding of a decrease in volume during the period of hypertension in others would seem to indicate that the elevation in pressure was not dependent upon an increase in the total amount of circulating blood.

While all animals developed cardiac hypertrophy, congestive failure did not occur, and in only 1 animal (Dog 34) was there an increase in total blood volume of the degree associated with congestive heart failure in humans.[5]

No anemia, even in the dog suffering from azotemia,.occurred.

The observed fluctuations in blood volume were greater than those found by Freeman and Page[1] in sympathectomized dogs.

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[5] Gibson, J. G., 2d, and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, **16**, 851.

## 10251

# Agar-Slant Tissue Cultures of Spotted Fever Rickettsiae.*

FLORENCE K. FITZPATRICK. (Introduced by Hans Zinsser.)

*From the Department of Bacteriology and Immunology, Harvard University Medical School.*

During June of 1938 our attention was called by the Massachusetts Department of Public Health to a patient on Cape Cod, a girl of 8 years, who was suffering from a disease clinically resembling spotted fever. The patient came from a region infested with ticks, and not far from East Brewster, where 2 cases of spotted fever were reported during 1937.[1] Blood was taken for us on the 8th day of illness by Dr. Rowley of the Hyannis Hospital, and 2 guinea pigs were inoculated intraperitoneally. One of these animals ran a fever, beginning the 5th day and continuing for 6 days, the temperature reaching 105.6°. Blood and spleen from the other guinea pig, taken on the 7th day (temperature 105.5°), were passed on to normal guinea pigs, the strain being maintained for 4 generations. A total of 13 animals was inoculated, all of them reacting with temperatures and 4 of them with a noticeable scrotal swelling, but without necrosis. Though there was no mortality, the animals were found to be immune when reinoculated with a virulent strain of

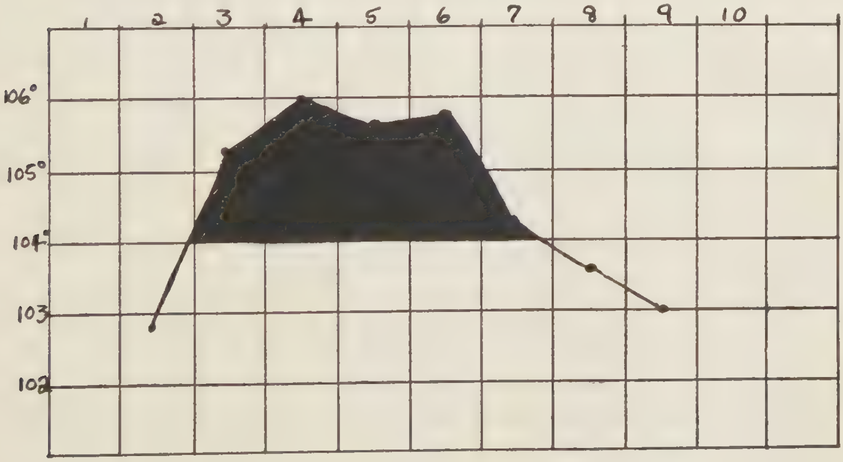

CHART 1.
Temperature curve of guinea pig inoculated with ½ a slant, 6th generation culture.

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* This work was supported in part by a grant from the Commonwealth Fund.

[1] *Rhode Island Med. J.*, 1937, **20**, 139.

spotted fever obtained through the courtesy of Dr. Dyer of the National Institute of Health.

Cultures were initiated with spleen and tunica by the agar slant method[2, 3] from one of the original guinea pigs. Almost all tubes showed good growth with some intranuclear Rickettsiae by the 10th to 14th day, at which time transfers were made to new slants, with mouse embryo as the fresh tissue. Growth has continued very rich through 10 generations, and transplants may now be made as early as the 6th day. Minced chick embryo may also be used with equally good results.

Guinea pigs inoculated with ½ a slant of the 6th generation mouse culture reacted as shown in Chart 1. Sections of tissues from these animals, examined for us by Dr. Henry Pinkerton, were characteristic of spotted fever. The cultures are still being maintained in series.

### 10252

## Ascorbic Acid Content of Milk of Various Species as Influenced by Ascorbic Acid Injection and Diet.

RUSSEL RASMUSSEN, RALPH BOGART AND L. A. MAYNARD.
*From the Laboratory of Animal Nutrition, Cornell University, Ithaca, New York.*

This paper reports studies of the ascorbic acid content of the milk of the ewe, cow, mare, and guinea pig. The determinations were made by the titration procedure, using 2,6-dichlorophenolindophenol. The samples were so handled in drawing them from the animal and thereafter as to exclude the light factor which Kon and Watson[1] found responsible for low values. A special microtitration method was devised for studying the guinea pig milk.

A ewe fed a ration of alfalfa hay and grain was milked twice daily for 9 weeks beginning the 12th day after lambing. The yields were recorded and samples were periodically taken for titration. During the last part of the experimental period the influence of ascorbic acid injection was studied. The results are presented in Fig. 1. The data reveal that during the period prior to ascorbic

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[2] Zinsser, H., Wei, H., and FitzPatrick, F., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 285.

[3] Zinsser, H., Wei, H., and FitzPatrick, F., *J. Exp. Med.*, in press.

[1] Kon, S. K., and Watson, M. B., *Biochem. J.*, 1936, **30**, 2273.

acid injection the values ranged from approximately 25 to 40 mg of ascorbic acid per quart, following a course suggesting an inverse relation to milk yield, as reported for the cow by Rasmussen, *et al.*[2] The intravenous injection of 5 g of ascorbic acid in 20 ml of sterile, distilled water caused a 50% rise in the ascorbic acid content of the milk in 12 hours, followed by a rapid drop to the initial value. A similar result was obtained by a second injection.

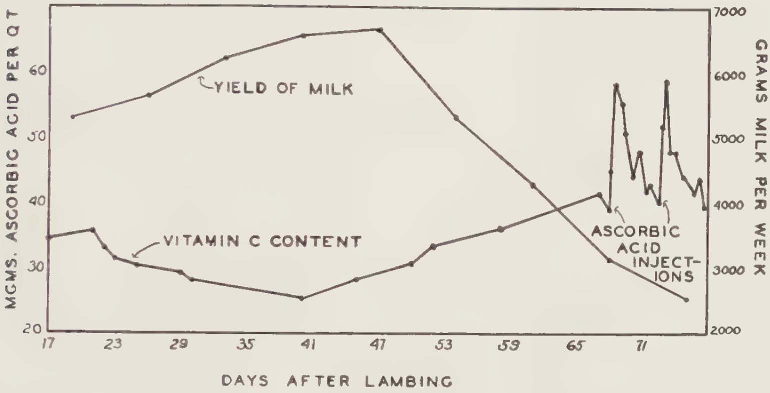

Fig. 1.

The effect of ascorbic acid injection was also studied with two cows, using 24 g in 100 ml of water in one case (Amy) and 12 g in 50 ml in the other (Cleo), with the results shown in Table I.

TABLE I.

| | Amy | | | Cleo | | |
|---|---|---|---|---|---|---|
| | Day | Milking | Ascorbic acid per quart, mg | Day | Milking | Ascorbic acid per quart, mg |
| Before injection | 1 | 8 P.M. | 19.2 | 1 | 8 P.M. | 17.8 |
| | 2 | 8 | 19.2 | 2 | 2 | 17.8 |
| | 3 | 8 | 19.2 | 2 | 8 | 17.9 |
| | 4 | 6 A.M. | 19.2 | 3 | 6 A.M. | 17.8 |
| | | 6:15 | Injection | | 6:15 | Injection |
| After Injection | 4 | 2 P.M. | 25.0 | 3 | 2 P.M. | 22.6 |
| | 4 | 8 | 30.8 | 3 | 8 | 25.5 |
| | 4 | 12 | 28.3 | 4 | 6 A.M. | 22.8 |
| | 5 | 6 A.M. | 27.1 | 4 | 2 P.M. | 19.4 |
| | 5 | 2 P.M. | 25.0 | 4 | 8 | 19.4 |
| | 6 | 6 A.M. | 21.9 | 5 | 6 A.M. | 17.2 |
| | 6 | 2 P.M. | 22.7 | 5 | 2 P.M. | 16.4 |
| | 6 | 8 | 22.7 | 6 | 2 | 16.4 |
| | 7 | 8 | 20.8 | 7 | 2 | 16.4 |
| | 11 | 2 | 18.1 | | | |
| | 11 | 8 | 18.9 | | | |

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[2] Rasmussen, Russel, Guerrant, N. B., Shaw, A. O., Welch, R. C., and Bechdel, S. I., *J. Nutrition*, 1936, **11**, 425.

These results support those obtained with the ewe that the injection of ascorbic acid causes an increase in its secretion in the milk. Recognizing that if the injection procedure should disturb the gland in such a way as to increase its permeability to the passage blood constituents any increased titer for ascorbic acid might be meaningless, the milk of the ewe and one cow was analyzed for lactose and chlorides before and after injection. The absence of any significant changes in the concentrations of these constituents indicated that the normal functioning of the gland had not been upset.

The ascorbic acid content of the milk of 6 mares nursing foals was determined beginning at foaling or shortly after and covering a period which included both barn and pasture feeding. A total of 52 samples was examined and the values ranged from 27 to 115 mg per quart. While variations were evident among individuals, the nature of the feed appeared to be an important factor concerned in the wide variations noted for a given mare. The values were markedly higher during periods of succulent pasture than when dried up grass or hay and grain provided the feed. This observation differs from those noted for cows (Riddell, *et al.*[3]). It is felt that definite conclusions regarding the significance of our values obtained with mares should await confirmation by biological studies, since, so far as we are aware, the titration procedure has not been checked with a biological assay in the case of mare milk.

Although we found that small amounts of copper will destroy the titer of mare milk, we also found that sunlight does not decrease this titer to the extent that it does that of cow milk. The latter observation is of interest in connection with the report of Hand, *et al.*,[4] that lactoflavin is the agent in cow milk responsible for the sensitivity of ascorbic acid to light. Our limited studies by the fluorometric method indicated that the lactoflavin content of mare milk is at least very low.

A study was made of the ascorbic acid content of the milk of guinea pigs differently fed as regards the vitamin. The milk of one animal which was receiving a diet of crushed oats, mixed hay, and a limited allowance of green grass gave values of 108, 110, and 112 mg per quart in successive determinations. The milk of a guinea pig receiving all the green grass she would consume gave a value of 397 mg. For another animal receiving the same diet the value was found to be 379 mg. A guinea pig, which received, in addition to green grass, approximately 40 mg of ascorbic acid daily beginning 17 days prior to parturition, secreted milk having a value of 711 mg per quart.

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[3] Riddell, W. H., Whitnah, C. H., Hughes, J. S., and Lienhardt, H. F., *J. Nutrition*, 1936, **11**, 47.

[4] Hand, D. B., Guthrie, E. S., and Sharp, P. F., *Science*, 1938, **87**, 439.

To investigate further the effect of diet upon the ascorbic acid content of the milk of guinea pigs, 5 pregnant animals were placed on a ration designed to be low in the vitamin, a few days before term. All gave birth to apparently normal young, but at the age of 6 days signs of scurvy were evident in the young and this was confirmed by the autopsy of one from each litter. One mother was autopsied also and the typical symptoms found. Two mothers were given 40 mg of ascorbic acid daily at this time while the diet of the other 2 remained unsupplemented. The addition of the vitamin to the diet of the mothers resulted within 6 days in a decrease in the symptoms in the young, while 2 of the young of the mothers which received no supplement died from scurvy on the 10th day, and those remaining alive revealed severe symptoms. The mothers on the unsupplemented diet showed moderate scurvy, while the other showed none. In another experiment 6 pregnant females were placed on the same basal diet, but were removed from their cages daily and allowed to eat all the fresh, green grass they would consume in 2 hours. This procedure was continued after the young were born, but the latter were not given access to the green feed. The young grew normally and showed no signs of scurvy under this regime, in contrast to the marked symptoms which were found in the previous experiment where the mothers were fed throughout on the diet low in the vitamin.

These preliminary studies with the guinea pig provide both chemical and biological evidence that the ascorbic acid content of its milk is influenced by diet. The values, as determined by titration procedure, are surprisingly high, compared to those of the other species studied. They are in accord with the figure of 29 mg per 100 ml reported for guinea pig milk by Houston, Kon and Henry.[5]

*Summary.* Values for ascorbic acid in ewe milk, determined periodically over the lactation period, ranged from 25 to 40 mg per quart. The intravenous injection of ascorbic acid resulted in a marked, temporary rise in the vitamin content of the milk of the ewe and cow. A study of 52 samples of mare milk gave values ranging from 27 to 115 mg per quart. The nature of the feed appeared to be a factor in the wide variations noted. Values obtained on guinea pig milk ranged from 108 to 711 mg per quart. Both chemical and biological evidence was obtained that the ascorbic acid content of the milk of the guinea pig is influenced by the nature of the diet.

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[5] Houston, J., Kon, S. K., and Henry, K. M., *Chem. Ind.*, 1938, **57**, 276.

### 10253

# Effect of Sorbitol and Sucrose on Cerebrospinal Fluid Pressure and Urine Output.

HENRY G. SCHWARTZ AND ROBERT ELMAN. (Introduced by Evarts A. Graham.)

*From the Department of Surgery and the Neurosurgical Laboratory, Washington University School of Medicine.*

The intravenous administration of hypertonic sucrose has been shown to have distinct advantages over glucose in promoting diuresis and reducing cerebrospinal fluid pressure.[1-4] Bullock, Gregersen, and Kinney[1] demonstrated experimentally that the reduction of cerebrospinal pressure lasted for 5-8 hours following injection of 3-8 g of sucrose per kg of body weight. No secondary rise was noted. In their animals, an active diuresis was induced, lasting about 3 hours, during which time the kidneys eliminated about 4 times as much fluid as was given. Masserman,[2] in a clinical study of 35 patients, found that the injection of more than 100 cc of 50% sucrose resulted in an initial rise in cerebrospinal fluid pressure, which persisted for 20-80 minutes; subsequently reduction of pressure occurred which lasted for 2½ hours or more. In his series, there was very little reduction following 100 cc of the 50% solution; more marked reduction occurred with 300 cc. In 5 out of 17 cases, after injection of 500 cc fall in pressure was followed by a mild secondary rise. Masserman found that within 4 hours, 63% of the sucrose was eliminated in the urine; in 24 hours, 78-92% could be recovered from the urine. It is believed that the efficacy of sucrose as compared with glucose, depends upon the fact that the former is almost completely eliminated by the body, without passing the hemato-encephalic barrier;[1] theoretically, therefore, a secondary rise of cerebrospinal pressure does not occur.

In cases of arterial hypertension, the use of sucrose has been found to promote diuresis and relieve symptoms of increased intracranial pressure.[3] Hahn, *et al.*,[4] have reported favorably on its use in the treatment of head injuries.

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[1] Bullock, L. T., Gregersen, M. A., and Kinney, R., *Am. J. Physiol.*, 1935, **112**, 82.

[2] Masserman, J. H., *Bull. Jchns Hopkins Hosp.*, 1935, **57**, 12.

[3] Murphy, F. D., Hershberg, R. A., and Katz, A. M., *Am. J. Med. Sci.*, 1936, **192**, 510.

[4] Hahn, E. V., Ramsey, F. G., and Kohlstaedt, K. G., *J. A. M. A.*, 1937, **108**, 773.

West and Burget[5] compared the diuretic effect of sorbitol and sucrose. The former was found to be a much more efficient diuretic than sucrose in 3 dogs. According to their description, the hexahydric alcohol, sorbitol, is non-toxic, has enormous water-binding capacity, is rapidly excreted by the kidneys after intravenous injection, and has 1.88 times the osmotic pressure of sucrose. Solutions of sorbitol are stable to heat sterilization. Sorbitol has been used effectively in anuria.[6]

In attempting to determine the relative merits of sorbitol and sucrose as agents for the reduction of cerebrospinal fluid pressure and for diuresis, we have run parallel experiments on 9 pairs of dogs. The animals weighed between 8 and 12 kg. In each experiment, anesthesia was induced with nembutal (0.5 cc/kg) intravenously; at hourly intervals thereafter an additional 0.5 cc was given subcutaneously, to assure fairly constant depth of anesthesia. Under aseptic precautions, a needle was introduced into the cisterna magna and connected to an Ayer manometer. A catheter was inserted into the bladder. Preliminary observations were made for periods from ½ to 1½ hours, until we were satisfied that a basic level of pressure was present. Subsequently, each of the pair of animals was given 50% sucrose and 50% sorbitol respectively. The substances were given intravenously in a dosage of 2½ cc of the 50% solution per kilo. In preliminary experiments we had found that too-rapid injection almost always resulted in an initial rise. This fact has been noted by others in connection with sucrose.[1, 3] One of us (H.G.S.) had also observed this when sucrose is given too rapidly to patients suffering from neurosurgical lesions with increased intracranial pressure. Accordingly, we have made the intravenous injections simultaneously at a speed of 7 cc/min.

The chart demonstrates the average results in 9 dogs following sucrose injection, and 9 following administration of sorbitol. Changes in cerebrospinal fluid pressure were recorded every 3 minutes. Urine output is charted on the basis of cc eliminated in half-hour periods, calculated on the basis of 10 kg of body weight.

It is apparent that sorbitol is a much more efficient agent than sucrose in reducing cerebrospinal fluid pressure and promoting diuresis in dogs. The peak of diuresis and pressure reduction is reached at about the same time within the first half-hour after administration. With sorbitol the basic level of spinal fluid pressure was reached after 2¾ hours and was followed by a slight secondary rise. The

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[5] West, E. S., and Burget, G. E., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 105.

[6] Strohm, J. G., *West. J. Surg. Obst. and Gyn.*, 1938, **46**, 200.

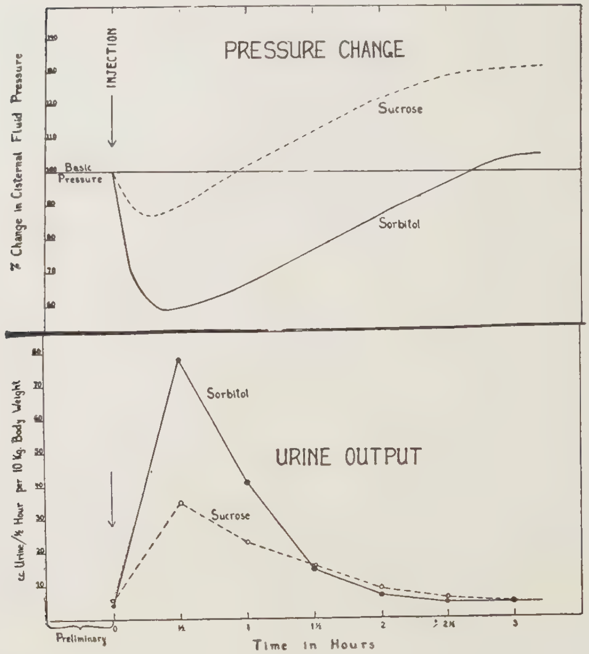

FIG. 1.

Average changes in cerebrospinal fluid pressure and urinary output following intravenous injection of 50% sucrose (- - - -) and 50% sorbitol (———-) in 9 pairs of dogs.

fall in pressure following sucrose lasted only 1¼ hours, and the secondary rise was pronounced. The presence of a secondary rise with sucrose in our experiments may seem surprising. It should be pointed out that the dosage which we have injected is much smaller than other investigators have used in animals.[1] This dosage, however, compares with the amounts of sucrose which we have used clinically. We, like Hahn, *et al.*,[4] have customarily given 100 cc doses of the 50% solution to patients. This amount is less than that given by Masserman[2] and Murphy, *et al.*[3]

*Summary.* Parallel experiments have been carried out on 9 pairs of dogs. 50% sorbitol, in a dosage of 2.5 cc/kg, injected intravenously at a rate of 7 cc/min causes a more marked and protracted fall in cerebrospinal fluid pressure, and a greater diuresis, than does an equivalent amount of sucrose.

### 10254

## Food Intake and Gastro-Intestinal Motility in the Albino Rat During Chronic CO Asphyxia.

Charles A. Patterson, Erma Smith* and H. B. Hale.
*From the Department of Zoology, Iowa State College, Ames, Iowa.*

Rats treated daily with carbon monoxide for increasing intervals of time become adapted to the gas and will withstand 0.34% for one hour after about 10 days. One hour in this percent CO causes the blood to become 70-80% saturated; it contains about 5% CO 24 hours after exposure; thus a state of chronic CO asphyxia is induced.[1] Illuminating gas containing CO, or pure CO may be used, the effects of illuminating gas being somewhat more pronounced.

*Food Intake* [Illuminating gas (I.G.)]. The following experiment was designed to measure the effects of chronic CO asphyxia on food intake. Thirty female albino rats were selected from 6 litters; litter mates of approximately equal weight were distributed in 2 groups. Fifteen animals were subjected to CO as described above and 15 were maintained as controls. The rats were caged individually and the food intake of all measured for periods ranging from 57-87 days. An adequate ration was fed *ad libitum*.[2] The results are shown in Table I.

TABLE I.
Food Intake During Chronic CO Asphyxia.

| | No. of animals | Body wt, g | | Food intake, mean g per day | |
|---|---|---|---|---|---|
| | | Initial | Final | | |
| Control | 15 | 173 | 240 | 8.28 ± .752 | Mean difference 1.98 |
| Gassed | 15 | 175 | 233 | 6.30 ± .253 | t = 2.4959* |

*Significant difference.

About 23% decrease in food intake is shown by the table. Diminished muscular activity and depressed oxidation probably account for the maintenance of body weight by the gassed rats.

*Gastro-Intestinal Motility.* (1) Effective peristalsis. In order to obtain a quantitative picture of the effects of CO on peristalsis a modification of Macht's technic was used.[3]

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* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Williams, I. R., and Smith, Erma, *Am. J. Physiol.*, 1935, **110**, 611.

[2] Lee, Milton O., *Am. J. Physiol.*, 1926, **78**, 246.

[3] Macht, D. I., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 1272.

Forty rats were selected from the stock colony and comparable animals paired for the tests, 2 being killed at each test. All were allowed to eat 3 g of the following mixture: bread, 25%; milk, 25%; hamburger, 48%; pure $Fe_2O_3$, 2%. Five minutes after eating, 20 of the rats were subjected to 0.34% CO in 1.43% I.G.-air mixture for 30 minutes. Forty-five minutes after eating the rats were decapitated and the distance of the dyed food from the stomach measured.

TABLE II.
Action of CO on Effective Peristalsis.

| | No. of animals | Distance traversed in cm mean | |
|---|---|---|---|
| Control | 20 | 35.0417 ± 2.374 | Mean difference 11.9167 |
| Gassed | 20 | 23.1250 ± 1.721 | $t = 4.0637$* |

*Highly significant difference.

It may be seen from the table that food is propelled about 33% more slowly in animals subjected to CO immediately after eating. This technic measures the inhibition during maximum saturation of the blood with CO.

(2). Egestion time. The time required for the first appearance of red fecal pellets following the test meal is a measure of the total time of passage through the alimentary canal. This interval we have termed the "Egestion time." For measurement of egestion time we have designed and constructed an excrementometer. (Fig. 1.)

A table, (T) with holes accommodates 6 twelve-inch funnels (F), on which rest cages (C), of ½-inch hardware cloth. Food, water and the test animals are maintained thus above a receiving disc (D), consisting of 6 concentric lanes divided into 64 compartments. The receiving disc is revolved by a kymograph (K), which makes one revolution in 16 hours. Hence the disc constitutes a timing unit, in which the maximum error is ±15 minutes.

Rats of similar age, weight and genetic history were arranged in 2 groups; the time elapsing between eating a test meal and the appearance of red fecal pellets was determined, using the excrementometer. Group I consisted of 40 rats; 20 were treated for one hour with 0.34% CO in 1.43% I.G.-air, immediately after eating the test meal described in (1). Group II consisted of 12 rats. Six were treated with 0.37% pure CO-air mixture immediately after eating 3 to 5 g of a test meal made up of 2% $Fe_2O_3$ in moistened, pulverized dog chow.

From Table III it may be seen that the egestion time is prolonged about 22% by treatment for one hour with 0.34%-0.37% CO.

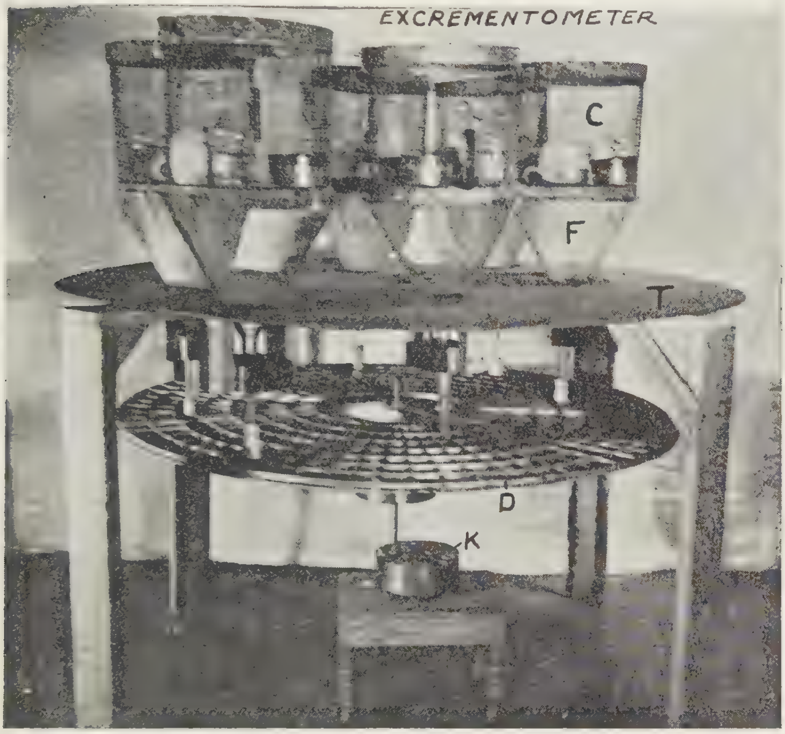

FIG. 1.

TABLE III.
Action of CO on Egestion Time.

| Group | | No. of animals | No. of determinations | Mean egestion time | Mean difference and t value |
|---|---|---|---|---|---|
| I | Control | 20 | 27 | 8.79 ± .505 | Mean difference 2.27 |
| | Gassed | 20 | 27 | 11.07 ± .655 | t = 2.1410* |
| II | Control | 6 | 27 | 6.89 ± .269 | Mean difference 1.49 |
| | Gassed | 6 | 27 | 8.38 ± .428 | t = 2.7510* |

*Highly significant difference.

Repeated tests using 6 animals (Group II) gave less variable results than single tests using 20 animals (Group I). Pure CO prolonged the egestion time less than I.G. containing an equal percentage of CO.

*Conclusions.* Chronic carbon monoxide asphyxia decreases food intake and inhibits gastro-intestinal peristalsis significantly.

## 10255

# Effect of Exertion on Vital Capacity of Normal Subjects.

ARNOLD IGLAUER AND MARK D. ALTSCHULE.

*From the Medical Research Laboratories, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston.*

Numerous studies have been made of the physiology of exercise, but comparatively little attention has been directed to the immediate effect of severe exertion on the vital capacity of normal subjects. Changes in the lungs, including reduction in vital capacity, are the rule in the dyspnea of congestive heart failure. Whether or not similar changes in the lungs occur in association with dyspnea due to severe exertion in normal subjects has not yet been established.

During the last century it was generally believed that dyspnea of exertion in healthy persons might be related to pulmonary engorgement. More recently, Bohr[1] and Gordon, Levine and Wilmaers[2] have mentioned this possibility. Available reports give conflicting data. Bohr[1] found that muscular activity, including running and work done while sitting and standing, decreased the vital capacity. Levine and Wilson[3] reported insignificant changes in the vital capacity of normal subjects who ran up and down stairs until breathless. Joannides[4] found that vital capacity was decreased in most of his normal subjects after exercising until dyspnea occurred. Gordon, *et al.*,[2] found definite diminution of the vital capacity of most of the runners after a 25-mile race. Harrison, Harrison, Calhoun and Marsh[5] observed little change in the vital capacity of normal subjects and patients with heart disease after mild exertion; their data are difficult to interpret since the nature and severity of the exercise were not noted. Because of these conflicting results and the variability in the conditions under which these studies were undertaken, the following investigation was made.

The vital capacity of untrained healthy adults was measured before and within 30 seconds after the ascent and descent of 4 flights of stairs as rapidly as possible. This exertion was sufficient to produce severe dyspnea which lasted for several minutes in all the subjects. All measurements were made in standing posture with the ordinary bell type of spirometer.

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[1] Bohr, C., *Deutsches Arch. f. klin. Med.*, 1907, **88**, 385.

[2] Gordon, B., Levine, S. A., and Wilmaers, A., *Arch. Int. Med.*, 1924, **33**, 425.

[3] Levine, S. A., and Wilson, F. N., *Heart*, 1919, **7**, 53.

[4] Joannides, M., *Arch. Int. Med.*, 1924, **33**, 145.

[5] Harrison, T. R., Harrison, W. G., Calhoun, J. A., and Marsh, J. P., *Arch. Int. Med.*, 1932, **50**, 690.

In 12 of the 15 subjects the vital capacity was slightly lower after exercise (Table I). No change of more than 4.7% occurred; the average change was less than 2%.

TABLE I.
Effect on Vital Capacity of Running Up and Down Stairs.

| | Vital capacity in liters | | Change after exercise | |
|---|---|---|---|---|
| Subject | Before exercise | After exercise | Liters | % of original value |
| 1 | 5.40 | 5.30 | —0.10 | —1.9 |
| 2 | 4.45 | 4.40 | —0.05 | —1.1 |
| 3 | 6.50 | 6.50 | 0 | 0 |
| 4 | 3.93 | 3.82 | —0.11 | —2.8 |
| 5 | 4.22 | 4.23 | +0.01 | +0.2 |
| 6 | 4.19 | 4.05 | —0.14 | —3.3 |
| 7 | 4.92 | 4.82 | —0.10 | —2.0 |
| 8 | 5.42 | 5.30 | —0.12 | —2.2 |
| 9 | 5.24 | 5.01 | —0.23 | —4.4 |
| 10 | 2.70 | 2.68 | —0.02 | —0.7 |
| 11 | 4.12 | 4.04 | —0.08 | —1.9 |
| 12 | 2.58 | 2.46 | —0.12 | —4.7 |
| 13 | 2.90 | 2.82 | —0.08 | —2.8 |
| 14 | 5.03 | 5.10 | +0.07 | +1.4 |
| 15 | 3.78 | 3.65 | —0.13 | —3.5 |

The slight changes found in the vital capacity of normal subjects after exercise are within the limits of error of the measurement. However, so many of the subjects showed changes in the same direction that it might be argued that the observed decreases are significant. The slight decreases are to be explained by the distress and discomfort associated with dyspnea. It is concluded, therefore, that no significant change in the vital capacity of normal subjects occurs after exercise which is strenuous enough to produce severe dyspnea. This is in accord with the findings of Levine and Wilson.[3]

The dyspnea of patients wth congestive heart failure is associated with a decrease in vital capacity which has been explained on the basis of congestion of the pulmonary circuit;[6] pulmonary congestion does not appear to be a factor in the dyspnea of exertion of normal persons. Confirmatory evidence as to the absence of pulmonary congestion in the dyspnea of exertion in normal subjects is the fact that the arterial blood oxygen saturation is not decreased after exercise severe enough to cause dyspnea in such subjects.[7, 8]

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[6] Drinker, C. K., Peabody, F. W., and Blumgart, H. L., *J. Exp. Med.*, 1922, **35**, 77.

[7] Himwich, H. E., and Barr, D. P., *J. Biol. Chem.*, 1923, **57**, 363.

[8] Cullen, G. E., Harrison, T. R., Calhoun, J. A., Wilkins, W. E., and Tims, M. M., *J. Clin. Inv.*, 1931, **10**, 807.

*Summary*. The vital capacity of normal subjects is not diminished by exercise severe enough to cause marked dyspnea. It is concluded that the dyspnea of exertion of normal subjects is not due to impaired pulmonary function consequent to congestion of the lungs.

## 10256

## The Urinary Pigments in Four Cases of Alcoholic Pellagra.

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Beckh, Ellinger and Spies[1] employed a very simple procedure for detecting and estimating porphyrin in the urine in 20 cases of pellagra. This consisted of extraction of the acidified urine with ether, after which the ether was extracted with a small amount of 25% HCl. The presence of a red or red violet color in this solution was considered due to porphyrin, and was estimated colorimetrically by comparison with a standard porphyrin solution.

In the present investigation the results of the B.E.S. (Beckh-Ellinger-Spies) test have been compared with the values for coproporphyrin obtained by means of a slight modification of the Fikentscher[2] method (estimation of red fluorescence of coproporphyrin in ultraviolet light, with the Zeiss stufenphotometer). Quantitative determinations were made in 3 of 4 cases of typical alcoholic pellagra.* In the first case but a single 24-hour urine sample was available prior to nicotinic acid therapy; this was examined qualitatively by the usual acetic and ether, followed by 5% HCl, extractions. The amount of porphyrin did not appear to be appreciably increased.

In a series of 5 normal individuals the range of coproporphyrin with the quantitative method employed, was 16-62.8$\gamma$ per day. In the 3 pellagra cases in whom determinations were made, the following results were obtained:

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[1] Beckh, W., Ellinger, P., and Spies, T. D., *Quart. J. Med.*, 1937, **30**, 305.

[2] Fikentscher, R., *Biochem. Z.*, 1932, **149**, 257.

* Two of these cases were on the medical service of the Johns Hopkins Hospital, Baltimore, and 2 were on the medical service of the Minneapolis General Hospital. The writer is indebted to Drs. Warfield Longcope and George Fahr, respectively, for access to the clinical data and urines of these cases.

| Case 2 | | | Case 3 | | | Case 4 | | |
|---|---|---|---|---|---|---|---|---|
| Date | 24-hour urine copro-porphyrin in $\gamma$ | B.E.S. Test | Date | 24-hour urine copro-porphyrin in $\gamma$ | B.E.S. Test | Date | 24-hour urine copro-porphyrin in $\gamma$ | B.E.S. Test |
| 6-12 | 65.7 | + | 8-25 | 241.0 | + | 9-25 | 101.1 | + |
| 6-13 | 218.6 | — | 8-26 | 283.1 | + | 9-26 | 232.3 | + |
| 6-14 | 109.3 | — | 8-27 | 358.8 | — | 9-28 | 452.4 | — |
| | nicotinic acid begun | | 8-28 | 425.0 | — | | nicotinic acid begun | |
| | | | 8-29 | 174.0 | — | | | |
| 6-15 | 81.6 | — | 8-30 | 264.9 | — | 9-30 | 537.1 | — |
| 6-16 | 140.0 | — | 8-31 | 263.8 | — | 10- 1 | 157.0 | — |
| 6-18 | 95.2 | — | 9- 1 | 299.2 | ? (faint color) | 10- 5 | 93.9 | — |
| 6-22 | 57.8 | — | | nicotinic acid begun | | 10-10 | 129.5 | — |
| 6-23 | 85.4 | — | | | | | | |
| | | | 9- 4 | 57.1 | — | | | |
| | | | 9- 6 | 99.7 | — | | | |
| | | | 9- 8 | 144.9 | — | | | |
| | | | 9-10 | 107.4 | — | | | |

It is seen that the amounts of coproporphyrin were distinctly increased prior to nicotinic acid therapy, decreasing significantly, although not into the normal range, thereafter. It should be pointed out that the persistently somewhat elevated values may be attributed readily enough to chronic alcoholism alone.[3, 4]

The above data fail to reveal any correlation between the amount of porphyrin as determined fluorimetrically, and the B.E.S. test. It will be noted that the test was positive when the 24-hour coproporphyrin was as little at 65.7$\gamma$, and negative when it was elevated to 537$\gamma$. Further study of urine samples exhibiting positive B.E.S. tests, revealed that the color was not due to porphyrin but to another red pigment which will be mentioned more fully below. If the ether extract, as obtained in the B.E.S. test, is first extracted with 5% HCl, this solution, although nearly or entirely colorless, will often be seen to exhibit faint porphyrin absorption at 550m$\mu$. If the ether is next extracted with 25% HCl a reddish pink color appears, but spectroscopic examination now fails to reveal any porphyrin absorption. From this it is quite clear that the B.E.S. test is not specific for porphyrins, although there is no doubt that large amounts of porphyrin, if present in the urine, would give rise to a positive test. The Beckh-Ellinger-Spies method is even less satisfactory as a quantitative procedure, since disturbing colors, particularly those due to urobilinogen derivatives, interfere. It was at first thought that mesobiliviolin might be present in sufficient amount to be productive of positive tests, but subsequent study of a number of urines rich in urobilinogen (the mother substance of mesobiliviolin) has shown that this is not the case.

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[3] Brugsch, J. T., and Keys, A., *Proc. Staff Mayo Clinic,* 1937, **12**, 609.

[4] Franke, K., *Z. f. klin. Med.,* 1936, **130**, 222.

Urine from the first case showed a positive B.E.S. test in Baltimore when the fresh urine was examined.† This was negative when the test was carried out in Minneapolis. It was noted that the toluene preservative was pinkish red.‡ More red pigment was extracted from the urine after acidification with glacial acetic acid. It was impossible to remove the red pigment from the combined ether-toluene fraction, by shaking with either 25% HCl or 10% $NH_4OH$. Crystals of the red pigment were readily obtained upon concentration of the ether-toluene solution. The yield was approximately 2 mg. The substance was sparingly soluble in ethyl acetate and was recrystallized from hot ethyl acetate, also from ethyl acetate and a relatively large volume of petroleum ether, in which the pigment is quite insoluble. The crystals were in the form of long needles (Fig. 1). Unfortunately, they sublimed gradually above 150°C, so that a melting point determination was excluded.

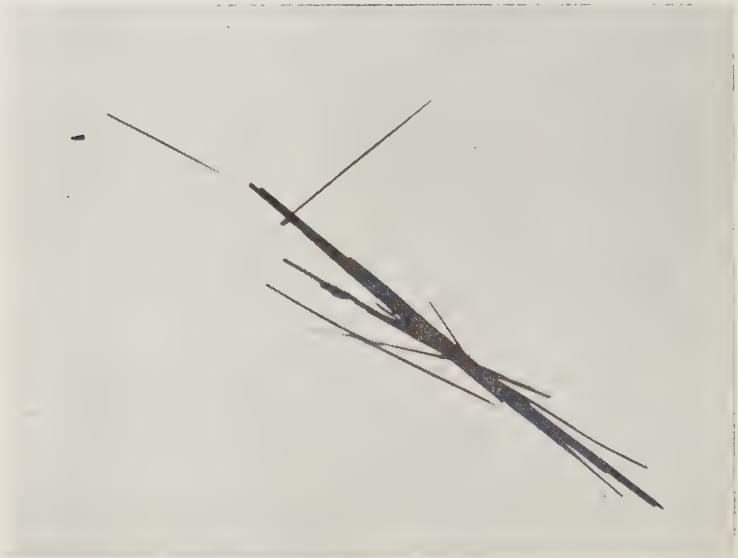

FIG. 1.
Crystals of red pigment from case 1. × 150.

The urine from Case 2 contained a very similar red pigment. Again the toluene preservative was pinkish, although the concentration of substance was not as great as in Case 1. Crystals were not obtained. A red pigment was also noted in the urine of Case 3, although in this instance the toluene preservative was not pink. In order to produce conditions similar to those for Cases 1 and 2, where the urines had been transported a long distance under toluene, a 24-hour urine sample from Case 3 was kept under toluene for 10 days, with frequent shaking daily. The toluene, however, did not become colored. The red pigment in Case 3 differed further in that it was extractable from ether by means of 25% HCl, although with difficulty.

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† The writer is indebted to Dr. W. Halsey Barker for reports of the B.E.S. tests on the fresh urines of cases 1 and 2, and for transmission of these urine samples to Minneapolis.

‡ Spies has observed that the toluene preservatives of pellagra urines, particularly after transportation, are often pink or reddish. Personal communication.

In spectroscopic character the red pigments noted in the first 3 cases were quite similar. In each instance there was broad, diffuse absorption, with poorly defined margins, in the region from 5700-5300Å. An ethyl acetate solution of the crystals from Case 1 exhibited absorption from 571-516mμ, with a slight break between 548-536; the redward half of this absorption was somewhat more intense. The absorption of this solution in the ultraviolet was photographed by Dr. Irwin Vigness of the Division of Biophysics in the University Hospital. This revealed well defined, although rather weak absorption at max. 2870Å. Similar absorption (max. 2900Å) was noted in an ethyl acetate extract from Case 3, but was not observed in such an extract from Case 2. This may have been explained simply on the basis of dilution, since the absorption in the most concentrated solution was relatively weak.

It is important to note that the red pigment was not observed in the urines of Cases 1, 2, and 3 after nicotinic acid therapy. This was true of toluene preservative, ether extract, and secondary 25% HCl fraction.

The urine from Case 4 exhibited a positive B.E.S. test in the first 2 days' collection. The ether extracts from 400 cc amounts of these urines, however, failed to show any pink or red color. This was also true of the subsequent 25% HCl extracts from the ether. Because of bad emulsions the process of ether extraction was much slower with the larger amounts and it could only be assumed that the pigment was labile and had faded during the manipulations.

Coproporphyrin III dimethyl ester was isolated from the total 8-day urine collection prior to nicotinic acid therapy in Case 3. The substance crystallized slowly in the rosettes of prisms characteristic of copro-III, and after 4 recrystallizations the melting point was sharp at 164-165°C. The yield was approximately 0.5 mg. The significance of this finding is not clear, but, at least insofar as the one case is concerned, indicates that the disturbance of pigment metabolism was similar to that known to exist in lead and arsenic poisoning.[5]

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[5] Watson, C. J., in *Oxford Medicine*, Chap. IX, p. 228.

Red urine has not been described as a symptom of pellagra, either endemic or alcoholic. The amounts of urinary porphyrin reported by Beckh, Ellinger and Spies were in many instances so large that the urine might have been expected to be red, since the amounts were as large as are often encountered in the wine red or dark red urines of idiopathic porphyria. This discrepancy is probably explained by the fact that the simple procedure they used is not specific for porphyrins and is not suitable for quantitative determination of the porphyrins. In the 3 cases discussed here, in which fluorimetric determinations were made, the per diem amount of coproporphyrin in no instance exceeded 0.6 mg (600$\gamma$), while Beckh, Ellinger and Spies reported 4 cases in which the amount was *over* 100 mg per liter, 3 in which the amount was between 10-100 mg, and 3 in which it was between 1-10 mg. In all of the present cases red pigments have been encountered differing from porphyrins or known hemoglobin derivatives. To what extent these pigments are related is not known. Considerable similarity in spectroscopic absorption was observed. The possibility must be considered that a common parent substance is altered variously in different urines and under different conditions, possibly undergoing esterification or conjugation. A change of the latter type was suggested particularly in the first of the present 4 cases, in which 25% HCl extracted a red pigment from the ether extract of the fresh urine, but later failed to remove it.

Prof. J. F. McClendon, Department of Physiological Chemistry, University of Minnesota, suggested that these red pigments might be indigo derivatives. Indirubin (indigo red, indipurpurin) exhibits similar absorption in xylene at 561 and 522 m$\mu$ (max.) and crystallizes in crimson needles which sublime without melting. The possibility is being investigated that this is the red substance which has been encountered in pellagra urines.

### 10257

## A Rapid and Sensitive Method for Bioassay of the Adrenal Cortical Hormone.

HANS SELYE AND VICTOR SCHENKER.

*From the Department of Anatomy, Histology and Embryology, McGill University, Montreal, Canada.*

In view of the ever growing interest in the physiology of the cortical hormone, it seemed of importance to develop a method which would allow the detection of minute amounts of this principle in tissues and body fluids. It is well known that animals, deficient in a certain hormone, are especially sensitive test objects for it. Use has been made of this observation for instance in methods for the bioassay of the pituitary hormones which can be detected most readily in hypophysectomized animals. As previous experiments have shown, there is a particularly acute need for cortical hormone during the alarm reaction, in fact, it appears that one of the most important, if not the most important function of the adrenal cortex is to make adaptation possible to such acute damaging agents as are capable of eliciting this reaction.[1, 2] It seemed advisable, therefore, to assay the cortical hormone in adrenalectomized rats during exposure to an alarming stimulus. Exposure to cold proved to be a stimulus which elicits an uncomplicated type of an alarm reaction, that is, the characteristic symptoms of damage as such (gastro-intestinal ulcers, hemoconcentration, hypoglycemia, etc.) appear without being significantly altered by changes due specifically to cold. We, therefore, decided to use adrenalectomized rats exposed to low temperature for the bioassay of the cortical hormone and our preliminary experiments soon showed that our supposition was correct and that under these conditions, minute doses of cortical hormone exert a marked effect and may readily be detected.

For such an assay we use nine male or female "hooded" rats weighing between 35 and 50 g, the optimum being 45 g, for each level of hormone concentration. We make it a rule to use 9 untreated adrenalectomized controls of the same size for each assay. Twelve to 24 hours after adrenalectomy, food and water are removed and the rats are placed in a refrigerator at a temperature of +2° - +5°C. At the same time, the 9 treated receive a subcutaneous injection of the hormone preparation dissolved in 0.2 cc of distilled water. Two more injections of the same type are given at 3-hour intervals, care being taken to make injections as rapidly as possible so that the animals are not kept outside the refrigerator too long. The total amount of the hormone preparation is thus administered in 3 doses within 6 hours. If the material which is to be assayed is toxic, one might administer it orally. The controls begin to show signs of hypoglycemia (convulsions and coma) within 4 to 5 hours, and most of them die between the sixth and the tenth hour, although occasionally some of the larger rats survive as long as 12 hours. The treated rats, on the other hand, survive much longer and show

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[1] Selye, Hans, *Arch. internat. Pharm. e. Ther.*, 1937, **55**, 431.

[2] Selye, Hans, Proc. Soc. Exp. Biol. and Med., 1938, **38**, 728.

no obvious signs of damage. We found it advisable to base our unit of the hormone on the difference between the survival rate of the controls and the treated animals, rather than on the absolute survival time of the treated animals alone, because in this case, variations in the temperature of the refrigerator are less likely to affect the result. *We regard as one unit the minimum amount of the cortical hormone which suffices to maintain at least 6 out of 9 experimental animals alive at the time when 6 of the 9 controls are dead.* Of course if a sufficient amount of material is available, statistically more significant results will be obtained if a larger number of rats is used, but even in this case, we would advise making the reading when two-thirds of the controls are dead and to consider as one unit the minimum amount which maintains alive at least two-thirds of the treated animals at this time. This comparative method of assaying has the further advantage of eliminating the factor of varying sensitivity to cold of rats from colonies normally kept at different temperatures and the influence of seasonal variations on the length of survival of adrenalectomized rats. The following examples of assays on Wilson's* preparation will illustrate the manner in which the bioassay is performed.

Twenty-seven female rats weighing 35-50 g were adrenalectomized between 8 and 10 o'clock in the evening. Next morning at 8 o'clock, 9 of them received 0.3 cc of the cortical extract subcutaneously, 9 others, 0.1 cc and the remaining 9 were not treated. All animals were placed in a refrigerator having a temperature of +2° - +5°C without food or water. The treated rats received 2 more injections of the same magnitude as the first after 3 and 6 hours respectively. The first control died after 6 hours and all controls were dead after 10 hours at which time, none of the injected rats had succumbed. This experiment showed that even as small a total dose, 0.3 cc is above the unit.

In a second experiment conducted in exactly the same manner, we had 4 groups, each consisting of 9 rats of the same size. The first group received a total of 0.3 cc, the second 0.1 cc and the third 0.03 cc in 3 divided doses. In order to make these injections accurate, we diluted the extract so that the above mentioned amounts were always contained in 0.2 cc of fluid. The first 2 controls died after 8 hours. After 10 hours, 6 out of the 9 controls were dead, while in each of the 0.1 and 0.03 cc groups only one animal was dead at this time. In the 0.01 cc group, 2 animals were dead out of the total of 9 which according to our definition would indicate that even this amount is above the unit, but since 2 others were dying at this time, we believe that as far as one can tell from an experiment performed on a relatively small number of rats, this amount of the Wilson preparation corresponds approximately to one unit according to the above definition.

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* We are greatly indebted to the Wilson Laboratories who, through the courtesy of Dr. David Klein, have generously supplied us with their adrenal cortical preparation.

From these experiments, it appears that a total amount of 0.03 cc of Wilson's preparation per rat is still demonstrable with this method.

Since not every laboratory would always have a sufficiently large number of "hooded" rats of this size, we assayed the total doses of 0.6, 0.3, and 0.1 cc given in 3 injections on hooded rats weighing 63-80 g and found them all to be above the unit. As we assumed that these larger rats would be less sensitive, we unfortunately did not assay as low a dose as 0.03 cc, but the experiment indicates that the minimum amount detectable in the 63-80 g rat does not differ significantly from that detectable in the 35-50 g animal if the reading is made when two-thirds of the controls are dead. It should be noted, however, that all animals survived several hours longer in this series.

The advantages of our method may be summarized as follows: The test is *specific* because it is based on the preservation of life in adrenalectomized animals exposed to an alarming stimulus during fasting. While salt treatment may also increase resistance in adrenalectomized animals which receive food, such treatment has been found to be entirely ineffective in the case of the fasting animal.[3] Fasting has the further advantage of making the results obtained in different laboratories more comparable as differences in the diet have been proven to exert a marked effect on the length of survival.

The test is *sensitive* for the amount just detectable with our method is 0.03 cc of Wilson's preparation per rat, while in other survival tests on fed rats such as that of D'Amour and Funk,[4] 0.5 cc of the same preparation has to be administered daily over a period of 10 days to obtain a positive test, that is, the total amount necessary per rat is 5 cc or 167 times as much as can be detected with our method.

The test is *rapid,* as it gives positive results within 8 hours at the most—and within no more than 24 hours, even if we count the time from adrenalectomy—while ordinary survival tests take 10 to 20 days.

The test is *simple* as it involves no complicated surgical technic, since adrenalectomy in the rat is much easier to perform than in other animals, and no chemical determination is necessary.

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[3] Selye, Hans, *Brit. J. Exp. Path.,* 1936, **17**, 234.

[4] D'Amour, Fred E., and Funk, Dorothy, *J. Pharm.,* 1938, **62**, 307.

The fact that any animal is used only for one test eliminates the danger of acquired resistance against the cortical hormone, such as has been described by Hartman, *et al.,*[5] and Toby and Lewis.[6] Since the assay is performed almost immediately after adrenalectomy, accessory cortical tissue, if present, has no time to undergo compensatory hypertrophy, so that in our experience no untreated control survived longer than 12 hours.

Several investigators advised the use of the body temperature of adrenalectomized animals as an index for cortical hormone assays either following exposure to cold[7, 8] or during the hypothermia induced by ether.[9] The reason why these testes never enjoyed great popularity is that the relationship between the dose of the cortical hormone and its temperature-maintaining effect is not as close as that between the dose of the hormone and its life-maintaining effect.

*Summary.* A method for the bioassay of the adrenal cortical hormone is described which allows of its detection in minute quantities. It is based on the maintenance of life of adrenalectomized rats weighing 35-50 g exposed to a temperature of +2° - +5°C. The hormone is administered subcutaneously in 3 doses with periods of 3 hours between injections. The unit is defined as the minimum amount necessary to maintain the life of two-thirds of the treated rats at a time when two-thirds of the untreated controls succumb. Because of the simplicity, sensitivity, and rapidity of this test, it appears to be particularly suitable for clinical and physiological studies necessitating the detection of the adrenal cortical hormone in tissues and body fluids in which only traces are present.

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[5] Hartman, Frank A., Lewis, L. A., and Toby, C. G., *Endocrinol.*, 1938, **22**, 207.

[6] Toby, C. G., and Lewis, L. A., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 352.

[7] Hartman, Frank A., Brownell, Katharine A., and Crosby, Alford A., *Am. J. Physiol.*, 1931, **98**, 674.

[8] Widström, G., *Acta Med. Scandinavica*, 1935, **87**, 1.

[9] Martin, Steven J., and Maresh, Frank, *Am. J. Physiol.*, 1933, **105**, 273.

## 10258 P

# Mechanism of Insulin Convulsions. I. Serum Electrolytes and Blood Sugar in Relation to Occurrence of Convulsions.

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The increasing evidence that potassium plays an essential rôle in carbohydrate metabolism,[1, 2] as well as in nervous activity,[3] suggests the possibility that the decrease of this element in the blood plasma following administration of insulin[4, 5] might be involved in the mechanism of insulin convulsions. The following is a brief report of experiments undertaken to explore this possibility.

The method used was to determine the level of K in the plasma immediately before and at various intervals after the administration of convulsive doses of insulin. Preparatory to their being subjected to the various experiments, dogs were kept on a standard diet for a fore period of several days, after which they were fasted for either 24 or 48 hours. Control blood specimens were obtained before each experiment was begun. Only those animals which had definite generalized insulin convulsions during preliminary tests were used for subsequent studies. The blood sugar, hemoglobin and plasma inorganic phosphorus were also determined. In a few instances changes in the serum calcium, lactic acid and sodium were likewise followed. The dosage of insulin varied somewhat from animal to animal, but in most of the experiments comparatively large doses (10 to 25 units per kg of body weight) were given because the results were found to be more regular with these than they were with smaller doses. The effect of insulin alone was determined 80 times in 43 dogs. In addition 8 experiments were carried out with continuous administration of K salts (chloride and citrate) in amounts sufficiently large to maintain the plasma K at normal levels while the blood sugar and inorganic phosphorus were reduced by the insulin. In other experiments phosphate, as well as K, was adminis-

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[1] McQuarrie, I., Thompson, W. H., and Anderson, J. A., *J. Nutrition*, 1936, **11**, 77.

[2] Silvette, H., and Britton, S. W., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 252.

[3] Holmes, E. G., *Annual Review of Biochemistry*, Stanford University Press, 1934, **4**, 435.

[4] Briggs, A. P. Koechig, I., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, 1923, **58**, 721.

[5] Harrop, G. A., and Benedict, E. M., *J. Biol. Chem.*, 1924, **59**, 683.

tered to prevent decrease in both of these inorganic ions during the period of hypoglycemia. Because administration of Na salts appears to accentuate the excretion of K and to have an effect on carbohydrate metabolism antagonistic to that of K, Na was administered in the form of chloride, lactate and citrate in different experiments. In order to elevate the plasma inorganic P without preventing the usual reduction in K and glucose, animals receiving insulin were made to breath atmospheres high in $CO_2$ in 11 experiments. The methods used for determination of the various blood constituents were as follows: glucose, Folin;[6] potassium, Shohl and Bennet;[7] inorganic phosphorus, Kuttner and Lichtenstein.[8]

The results may be summarized as follows: Although the plasma K and inorganic P values were decreased in all experiments following administration of insulin alone, there was no definite relationship between the absolute level of either substance and the occurrence of convulsions. In contrast with the findings in relationship to the hypoglycemic curve, there was no evidence of a tendency for convulsions to occur at the lowest point on either the K or the P curve. Administration of glucose caused a prompt return of the plasma K to normal without raising the inorganic P. Administration of K salts to prevent decrease in the plasma K did not prevent insulin convulsions so long as it did not prevent a fall in the blood sugar to the previously determined convulsive level. In 2 such experiments convulsions did not occur, but in neither of these did the blood glucose fall below 30 mg per 100 cc. When the plasma inorganic P was elevated or was maintained at normal levels by administration of $PO_4$ salts or by having the animal breath an atmosphere containing between 5% and 10% $CO_2$ plus 20% to 80% $O_2$, convulsions still occurred as in control experiments soon after the blood sugar had fallen to between 15 and 25 mg per 100 cc. Administration of NaCl appeared to hasten the onset of convulsions in one animal but in three others had no effect on their occurrence. Sodium lactate appeared to prevent the occurrence of a convulsion in one experiment in spite of a decrease in the blood sugar to a level slightly below that found just prior to a convulsion in the control experiment. In 2 other essentially identical experiments, however, this organic salt of sodium had no inhibiting effect. The effects of sodium citrate were almost identical with those of sodium lactate. The *conclusion* drawn from these experiments is that there is no direct relationship between the level of plasma K or inorganic P and the occurrence of insulin convulsions. Glucose was the only blood constituent that appeared to be of significance in this connection.

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[6] Folin, O., *J. Biol. Chem.*, 1929, **82**, 91.

[7] Shohl, A. T., and Bennet, H. B., *J. Biol. Chem.*, 1928, **78**, 643.

[8] Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, 1930, **86**, 671.

### 10259 P

## Mechanism of Insulin Convulsions. II. Effects of Varying Partial Pressures of Atmospheric $O_2$, $N_2$ and $CO_2$.

IRVINE MCQUARRIE AND MILDRED R. ZIEGLER.
*From the Department of Pediatrics, University of Minnesota, Minneapolis.*

In connection with a study on the relationship between the levels of potassium, inorganic phosphorus and glucose in the blood and the occurrence of insulin convulsions[1] dogs were subjected to atmospheres varying greatly in their partial pressures of $O_2$, $N_2$ and $CO_2$. The finding by Glickman and Gellhorn[2] that rats subjected to lowered $O_2$ tension (air in low pressure chamber at 460 mm Hg) became more sensitive to insulin and had convulsions sooner than they did in ordinary air, suggested the use of induced anoxic anoxia as an additional means of modifying our experimental conditions. The fact that the convulsions of epilepsy[3] and those of hypoparathyroidism can not infrequently be prevented or aborted by the subject's breathing a gaseous mixture containing 10% $CO_2$ and 90% $O_2$ further suggested the desirability of investigating the influence of such alterations in the air breathed on the various constituents of the blood and on the occurrence of convulsions following the administration of insulin.

The method used was that of determining the effects of insulin administration, first, when the experimental animal was allowed to breath room air, and again, one week later when the same animal was kept in an ordinary oxygen tent containing $O_2$, $N_2$ and $CO_2$ at different partial pressures. The insulin was given subcutaneously in large single doses (10 to 25 units per kilo of body weight). In addition to the preliminary control experiments performed on all animals, the following studies were carried out: 17 experiments with low $O_2$ percentages; 4 with high $O_2$; 4 with high $O_2$ plus high $CO_2$; 4 with low $O_2$ plus high $CO_2$, and 10 with high $CO_2$ plus 20% $O_2$.

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[1] Ziegler, M. R., and McQuarrie, Irvine, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 142.

[2] Glickman, N., and Gellhorn, E., *Am. J. Physiol.*, 1938, **121**, 358.

[3] Lennox, W. G., and Cobb, S., *Med.*, 1928, **7**, 105.

The specific effects of the various gaseous mixtures *per se* were determined before insulin was administered in some instances. The total pressure within the tent was always equal to the outside atmospheric pressure, variations in the partial pressures of the different gases being produced by admitting gas from special tanks. As a special check on the composition of the gaseous mixture within the tent, $O_2$ and $CO_2$ were determined at 30-minute intervals by the Orsat method. Blood samples were obtained at hourly or 2-hourly intervals without removing the animal from the tent. The technical procedures used for determination of the various blood constituents were the same as those referred to previously.[1]

The results may be summarized as follows: Breathing an atmosphere with oxygen reduced to between 5% and 12% caused marked hyperglycemia and a simultaneous reduction in both the K and the inorganic P of the plasma without administration of insulin. Hemolysis frequently occurred after prolonged experiments involving the lower percentages of $O_2$. High $CO_2$ (15%) alone caused hyperglycemia accompanied by a significant increase in plasma inorganic P. The K of the plasma was little influenced by the animal's breathing a high percentage of $CO_2$. The effects of breathing the various gaseous mixtures used on the occurrence of insulin convulsions were as follows: Breathing high partial pressures of $O_2$ (60% to 76%) or partial pressures of $CO_2$ between 5% and 10% or a combination of these had no definite effect, convulsions occurring at approximately the same time after the insulin injection as they did in room air. Low $O_2$ pressures (4.5% to 9.2%), however, tended to prevent convulsions entirely or to greatly prolong the period between the time of insulin administration and the occurrence of convulsions. In a few instances reduction of the $O_2$ to only 12% had a similar inhibiting effect. Convulsions did not occur in many of the low-oxygen experiments, even when the degree of hypoglycemia was similar to that found to precede the onset of convulsions in the corresponding control tests in ordinary air. The plasma K and inorganic P were reduced to approximately the same degree when the animal was kept in the low $O_2$ atmosphere after receiving insulin as they were in ordinary air. The effect of breathing gaseous mixtures which were very low in $O_2$ and high in $CO_2$ was to inhibit the convulsive tendency. An atmosphere containing 15% $CO_2$ plus 20% $O_2$ had a similar effect even when the blood sugar was reduced well below the previously determined convulsive level. In the latter type of experiment, the inorganic P of the plasma was greatly elevated while the K was reduced along with the blood sugar. Convulsions occurred in spite of the 15% $CO_2$, when the $O_2$ in the tent was increased to 80%. That the nervous system is not rendered entirely insensitive to convulsive agents by the low $O_2$, high $CO_2$ atmospheres is indicated by the fact that we were able to induce typical metrazol convulsions within the usual time under these conditions.

It may be concluded tentatively from the foregoing experiments that marked lowering of the $O_2$ content or marked increase in the $CO_2$ content of the respired air tends to inhibit rather than to accelerate insulin convulsions in the normal, fasting dog under the conditions described. While severe hypoglycemia appears to be the most important known causative factor, these experiments indicate that some additional physiological disturbance is involved in the mechanism of insulin convulsions.

## 10260 P

## Inhibition of Growth of Rat by Oral Administration of Methylcholanthrene. Effects of Dietary Cystine and Methionine Supplements.*

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In the present communication it is desired to report preliminary results obtained in studies of the effects of certain carcinogenic substances on sulfur metabolism. The experimental approach employed in the first series of investigations is similar to that which has resulted in the demonstration that bromobenzene,[1] cholic acid,[2] naphthalene,[3] and iodoacetic acid[4] exert an inhibiting effect on the growth of the young white rat when any one of these compounds is incorporated into a basal diet of relatively low protein content. The addition of either l-cystine or dl-methionine to the basal diet containing either bromobenzene, cholic acid, naphthalene, or iodoacetic acid resulted in a prompt stimulation of growth, despite the continued presence of the foreign organic compound in the diet. It was suggested that these organic substances are capable of producing a specific deficiency in the sulfur-containing amino acids, probably through the requirements of the organism for organic sulfur, in the form of cystine or methionine, for detoxication mechanisms. This conclusion is supported by the observation that inorganic sulfate, amino acid nitrogen in the form of a casein digest, and organic sulfur in the form of that found in taurine will not stimulate the growth of animals stunted by the administration of either bromobenzene, cholic acid or iodoacetic acid.

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* This investigation was aided by a grant from the Hubbard-McCormick Clinical Cancer Research Fund.

[1] White, A., and Jackson, R. W., *J. Biol. Chem.*, 1935, **111**, 507.

[2] White, A., *J. Biol. Chem.*, 1935, **112**, 503.

[3] Stekol, J. A., *J. Biol. Chem.*, 1937, **121**, 87.

[4] Simon, E. E., and White, A., *J. Biol. Chem.*, 1938, **123**, cix.

In the present studies, male rats at weaning have been placed on a basal diet of the following composition: casein† 6, starch 50, lard 24, sucrose 15, cod liver oil 1, and inorganic salts‡ 4%. In addition, each animal received a daily supplement of 400 mg of yeast.§ When the rats reached a body weight of 75 to 85 g, methylcholanthrene|| was incorporated into the basal diet. After a number of preliminary

TABLE I.
The Effect of Methylcholanthrene-containing Diets on the Growth of the Rat.

| Rat No. | Diet | Avg daily wt change, g | Avg daily food consumption, g |
|---|---|---|---|
| 2 | Basal | +1.6 | 4.8 |
| | " + methylcholanthrene* | +0.5 | 5.5 |
| | " + " + methionine | +1.4 | 5.8 |
| 5 | " | +1.4 | 6.1 |
| | " + methylcholanthrene | +0.6 | 5.9 |
| | " + " + glycine | +0.5 | 6.2 |
| 7 | " | +1.8 | 6.4 |
| | " + methylcholanthrene | +0.6 | 6.4 |
| | " + " + sodium sulfate | +0.8 | 7.3 |
| 8 | " | +1.8 | 6.8 |
| | " + methylcholanthrene | +0.3 | 6.0 |
| | " + " + taurine | +0.5 | 5.9 |
| 11 | " | +1.5 | 5.5 |
| | " + methylcholanthrene | +0.4 | 6.1 |
| | " + " + cystine | +1.5 | 5.8 |

*All of the methylcholanthrene-containing diets in this table had 60 mg of the hydrocarbon added for each 100 g of the basal ration. In experiments now being conducted, it has been observed that increasing the methylcholanthrene concentration to 70 mg per each 100 g of the basal diet results in an average daily growth increment of approximately 0.1 g. The effects of methionine or cystine supplements can thus be made even more striking.

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† Casein No. 453, Casein Company of America, Inc.

‡ Osborne and Mendel salt mixture (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, **37**, 572).

§ Product of the Northwestern Yeast Company.

|| Purchased from the Eastman Kodak Company.

trials, it was observed that when 60 mg of methylcholanthrene were incorporated into each 100 g of the basal diet, growth is inhibited. Although the rats do continue to grow, the rate of weight increase is markedly less than that observed in control animals ingesting the basal ration alone.

The incorporation of l-cystine (400 mg) or dl-methionine (500 mg) into each 100 g of the basal diet containing the methylcholanthrene results in a prompt stimulation of growth, with a resulting daily weight increment approximating that observed on the basal diet alone. On the other hand, supplements of glycine (500 mg), taurine (500 mg), or anhydrous sodium sulfate (500 mg), each added to 100 g of the basal diet containing methylcholanthrene, produced no increase in growth rate. Some typical data are presented in Table I. It appears, therefore, that cystine and methionine exhibit a specific ability to overcome the growth-inhibitory effect of methylcholanthrene. These results suggest that methylcholanthrene may produce a deficiency in the sulfur-containing amino acids, possibly by virtue of the involvement of these amino acids in the detoxication of the hydrocarbon. The metabolic fate of methylcholanthrene is being studied in order to obtain further evidence bearing on this suggestion, and similar methods are being extended to other carcinogenic substances.

### 10261

## "Acid" Phosphatase and Functional Activity of the Prostate (Man) and Preputial Glands (Rat).

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*From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.*

While investigating the source of marked outpourings of "acid" phosphatase in the urine of adult men, Kutscher and Wolbergs[1] discovered that normal adult prostate tissue is extremely rich in a phosphatase, apparently specific,[2] with optimal activity at approximately pH 5.0. At about the same time, Moore and Hanzel[3] noted that prostate tissue extracts split off inorganic phosphorus from sodium nucleinate, a "nuclease" effect attributable to nucleotidases, which are now classified as phosphatases.[4, 5] Prostate phosphatase has since been found also[6, 7] in the primary tumor, at the site of distant metastases and in the blood serum of patients with metastasizing carcinoma of the prostate gland. The implications of these observations have been considered elsewhere.[6, 7]

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[1] Kutscher, W., and Wolbergs, H., *Z. f. physiol. Chem.*, 1935, **236**, 237.

[2] Kutscher, W., and Wörner, A., *Z. f. physiol. Chem.*, 1936, **239**, 109.

[3] Moore, R. A., and Hanzel, R. F., *Arch. Path.*, 1936, **22**, 41.

The prostate gland of children, we find, contains little "acid" phosphatase (4.5 units at birth, 1.5 units at 4 years, 73.0 units at 13 years, per g fresh tissue). But adult prostate glands, though varying widely in phosphatase activity at pH 4.9 (from 2,284 to 522 units per g fresh tissue, in our series) consistently exceed by far the "acid" phosphatase activity of liver, kidney, duodenal mucosa and bone (Table I)—organs generally regarded as so rich in phosphatase as to imply some significant rôle of the enzyme in their respective functions. The presence in adult prostate tissue and in the semen of such high concentrations of this active enzyme (with optimal pH approximating that obtaining in the adult vagina) would suggest, similarly, that prostate phosphatase exercises some significant function in reproduction.

TABLE I.

Comparison of "Acid" and "Alkaline" Phosphatase Activity of Prostate Tissue with That of Other Tissues of the Adult Human.

(Phosphatase activity expressed in units/g fresh tissue.)

pH 4.9: *M*/200 monophenylphosphate substrate; *M*/10 citrate buffer; 37°C; 1 hr.

pH 9.0: *M*/200 monophenylphosphate substrate; *M*/20 Na veronal buffer; 37° C; 1 hr.

| | | Prostate pH | | Kidney pH | | Liver pH | | Duodenum pH | | Vertebra pH | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Case | Age | 4.9 | 9.0 | 4.9 | 9.0 | 4.9 | 9.0 | 4.9 | 9.0 | 4.9 | 9.0 |
| 1 | 43 | 522 | 1.3 | 4.6 | 2.9 | 1.6 | 1.2 | 3.1 | 11.3 | 2.5 | 8.8 |
| 2 | 45 | 792 | 0.8 | 2.4 | 6.7 | 2.4 | 2.1 | 0.9 | 5.9 | 1.9 | 3.0 |
| 3 | 45 | 2,284 | 0.9 | 3.1 | 2.3 | 2.6 | 10.6 | 0.8 | 4.0 | | |

An attempt to investigate this function further in experimental animals gave unexpected results. Repeated determinations at pH 4.9 revealed little phosphatase activity in the prostate gland of the sexually-mature dog (35.6 units per g fresh tissue), cat (2.8 units), rabbit (1.9 units), guinea pig (3.9 units), and rat (2.0 units).* Rat

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[4] Folley, S. J., and Kay, H. D., *Ergebn. d. Enzymforsch.*, 1936, **5**, 159.

[5] Bredereck, H., *Ergebn. d. Enzymforsch.*, 1938, **7**, 105.

[6] Gutman, E. B., Sproul, E. E., and Gutman, A. B., *Am. J. Cancer*, 1936, **28**, 485.

[7] Gutman, A. B., and Gutman, E. B., *J. Clin. Invest.*, 1938, **17**, 473.

* These results are in agreement with Wolbergs' recent findings[8] that unlike man and the apes, "in the steer, dog and several other animals, only very small amounts of phosphatase could be detected" (in the prostate gland).

[8] Quoted by Kutscher, W., and Pany, J., *Z. f. physiol. Chem.*, 1938, **255**, 169.

prostates, unlike the prostate glands of these other animals, were found to contain considerable amounts of "alkaline" phosphatase: 54.8 to 15.5 units per g fresh tissue, at pH 9.0, in animals with liver phosphatase not exceeding 3.6 units at that pH. But no significant phosphatase activity was found in adult rat prostates buffered at more acid levels, as shown in the following example: 3.2 units at pH 7.3, 3.9 units at pH 5.5, 3.1 units at pH 4.9, 0.1 unit at pH 2.2.

Rats were treated with appropriate steroids known to cause more or less specific proliferation of certain cellular components of the rat prostate gland.[9]

A. (1) Mature rats were injected with 2 mg of testosterone propionate twice at 4-day intervals, then killed after 5 to 9 days. The prostates showed 2.4 mean units of phosphatase activity per g fresh tissue at pH 4.9; 30.3 units at pH 9.0. (2) The prostate glands of immature rats treated in the same way yielded 2.8 mean units per g fresh tissue at pH 4.9; 27.8 units at pH 9.0.

B. Mature rats were injected with 200 rat units of estradiol benzoate in oil twice at 3-day intervals, killed 4 days later. The prostates showed mean values of 2.4 units per g fresh tissue at pH 4.9; 49.4 units at pH 9.0.

C. Immature rats were injected with 100 rat units of Follutein (Squibb) 3 times at 2-day intervals, killed 3 days later. The prostates showed mean values of 2.3 units per g fresh tissue at pH 4.9; 49.0 units at pH 9.0.

Although the enlargement of accessory sex glands caused by these agents resulted in some increase in the total content of "acid" prostate phosphatase, it will be noted that the "acid" phosphatase content per g fresh prostate tissue was not significantly elevated.

Other sources of "acid" phosphatase in the adult male genital tract of the rat were then sought. Little phosphatase activity at pH 4.9 was found in the testis (1.1 to 4.2 units per g fresh tissue), seminal vesicles (0.4 to 2.8 units) and Cowper's glands (0.9 to 1.6 units), even after injection of the steroids mentioned; and only testis showed significant values at pH 9.0 (12.2 to 18.6 units). On the other hand, rat preputial glands proved unexpectedly rich in "acid" phosphatase. At pH 4.9, 27.4 to 104.0 units per g fresh tissue were found, including a value of 43.2 units in a sexually-immature rat of 4 weeks. The steroid-treated animals showed no definite deviation from the controls, per g preputial gland, but the increased size of the glands caused an increase in total preputial gland phosphatase. The pH-activity curve of this "acid" phosphatase approximates that of human prostate phosphatase. Both are inhibited by sodium fluoride and by propyl alcohol, neither is accelerated by Mg ion: an extract of rat preputial gland with 102.5 units of "acid" phosphatase activity showed 65.0 units with *M* propyl alcohol; 7.0 units with *M*/200 NaF, and 99.0 units with *M*/50 $MgCl_2$. Rat preputial glands, like rat prostate glands, also contain significant amounts of "alkaline" phosphatase (11.6 to 50.1 units per g fresh tissue at pH 9.0).

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[9] Bühler, F., *Z. f. d. ges. exp. Med.*, 1938, **104**, 249.

These results suggest another approach to problems of the accessory sex glands. It is interesting to note in this connection that Disselhorst long ago.[10] on purely morphologic grounds, postulated a specific function of the rat preputial glands.

*Methods.* The ground glands were usually extracted with 10 times their weight of distilled water for 24-48 hours in the refrigerator. Further dilution of the extract as high at 1:500 was necessary for optimal hydrolysis in the cases of human prostate and rat preputial glands. Phosphatase activity at pH 4.9 was determined by the adaptation of the King and Armstrong method[11] described elsewhere.[7] The unit employed in this study is defined as that degree of phosphatase activity which at pH 4.9 and 37°C will liberate from the specified buffer-monophenylphosphate substrate solution 1 mg of phenol in 1 hour.

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## 10262 P

## Effect of Calcium in Quantitative Determination of Prothrombin.*

JOHN K. STEWART AND FREDERICK J. POHLE. (Introduced by W. S. Middleton.)

*From the Departments of Clinical Pathology and Medicine, University of Wisconsin Medical School.*

Recent investigations,[1, 2] indicate that satisfactory methods for the quantitative determination of prothrombin have been developed.

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[10] Disselhorst, R., in Oppel's *Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbeltiere,* 1904, **4**, 280.

[11] King, E. J., and Armstrong, A. R., *Canad. M. A. J.,* 1934, **31**, 376.

* This study has been aided in part by a grant from the Wisconsin Alumni Research Foundation.

[1] Quick, A. J., *J. A. M. A.,* 1938, **110**, 1658.

[2] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.,* 1936, **114**, 667.

These methods are dependent on the original suggestion of Quick, Stanley-Brown and Bancroft[3] that the coagulation time of oxalated plasma, when mixed with an excess of thromboplastin and then recalcified is a direct measure of the prothrombin content of the plasma. Subsequent studies have led some investigators to conclude that the defect in the coagulation of the blood in certain cases of jaundice is due to a deficiency of prothrombin.

The methods followed in the present studies were essentially the same as those described by Quick.[1, 4] The prothrombin time was determined on 0.1 cc portions of oxalated plasma in clean, dry 100 x 13 mm test tubes in a water bath at 37.5°C. An excess of thromboplastin was supplied by the addition of 0.1 cc of a freshly prepared saline suspension of an acetone extract of rabbit brain. Theoretically the thromboplastic preparation served to convert all of the prothrombin to thrombin. Upon recalcification with 0.1 cc of a 0.025 M calcium chloride solution, which was considered to be the optimal amount of calcium,[1] it was observed that the results were extremely variable. Furthermore, it was usually impossible to obtain a fibrin clot in normal plasma in from 12 to 13 seconds[1] unless the concentration of calcium was altered.

Eleven solutions of calcium chloride varying in concentration from 0.1 M to 0.000625 M were prepared. The effect of each of these solutions as a recalcifying agent in the determination of prothrombin was studied on plasma obtained from 20 normal individuals. In each instance the results were entirely similar. The observations in one typical case are shown in Table I. The data

TABLE I.
Effect of Calcium Concentration on Prothrombin Time of Normal and Pathological Subject.

| | Coagulation time in seconds | |
|---|---|---|
| Molar conc. of CaCl | Normal | Jaundice |
| .1 | 48 | 340 |
| .05 | 24 | 73 |
| .033 | 19 | 49½ |
| .025 | 15½ | 40 |
| .02 | 13½ | 38 |
| .015 | 13 | 32 |
| .01 | 12 | 32 |
| .005 | 11 | 29 |
| .0025 | 10 | 29 |
| .00125 | 15 | 50 |
| .000625 | 38 | 124 |

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[3] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sc.*, 1935, **190**, 501.

[4] Quick, A. J., personal communication.

obtained on one of 4 cases of obstructive jaundice with a hemorrhagic diathesis are presented in the table for comparison The results clearly show that an excess of calcium as well as an insufficient amount of calcium prolonged the time required for the coagulation of normal and pathological plasma. The optimal amount of calcium required varied among normal individuals. Usually recalcification with 0.1 cc of a 0.0025 M calcium chloride solution resulted in a minimal coagulation time of 10 seconds. However, in certain instances a minimal coagulation time occurred when recalcification was carried out with 0.01, 0.005, and 0.00125 M calcium chloride solutions.

It is well known that an excess of any neutral salt preserves the fluidity of the blood. Horne,[5] Sabbatini,[6] Rettger,[7] and Stassano and Daumas[8] observed the anticoagulant properties of large amounts of calcium salts.

If the quantitative determination of prothrombin is to be reliable there must be no variables except the prothrombin. The present studies indicate that calcium is a variable and that in order to obtain a minimal coagulation time (true prothrombin time) the optimal amount of calcium necessary for recalcification must be determined in each instance.

## 10263

## Use of Cyanide in the Determination of Ascorbic Acid.

M. CUSHMAN AND ALLAN M. BUTLER. (Introduced by James L. Gamble.)

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Though blood plasma or serum inhibits the catalytic oxidation of ascorbic acid as shown by De Caro and Giani[1] and Mawson,[2] Barron, Barron, and Klemperer[3] found that this protective property of plasma did not completely prevent such oxidation. Kellie and

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[5] Horne, R. M., *J. Physiol.*, 1896, **19**, 356.

[6] Sabbatini, L., Abstr. in *Mosso's Arch. ital. de biol.*, 1901, **36**, 416.

[7] Rettger, L. J., *Am. J. Physiol.*, 1909, **24**, 406.

[8] Stassano, H., and Daumas, A., *Compt. rend. Acad. Sc.*, 1924, **150**, 937.

[1] De Caro, L., and Giani, M., *Z. f. physiol. Chem.*, 1934, **228**, 13.

[2] Mawson, C. A., *Biochem. J.*, 1935, **29**, 569.

[3] Barron, E. S. G., Barron. A. G., Klemperer, F., *J. Biol. Chem.*, 1936, **116**, 563.

Zilva[4] and Barron, De Meio, and Klemperer[5] reported complete inhibition of the catalytic oxidation of ascorbic acid by the addition of potassium cyanide. Pijoan, Townsend and Wilson[6] reported that the concentration of ascorbic acid in plasma and in the metaphosphoric acid filtrate of plasma decreased significantly over short time intervals. To prevent such loss during plasma ascorbic acid determinations Pijoan and Klemperer[7] advocated the addition of approximately 1 mg of potassium cyanide per cc of blood. Pijoan and Eddy[8] in attempting to prevent the loss of ascorbic acid during the analysis of whole blood added as much as 2 mg of potassium cyanide per cc of whole blood. Mindlin and Butler[9] added 1 drop of 5% KCN to 5 cc of blood (approximately 0.6 mg of potassium cyanide per cc) in the determination of plasma ascorbic acid. Friedman, Rubin, and Kees[10] showed that in the absence of cyanide there was no significant loss of reducing power in plasma standing in an ice box for 3 hours. Moreover, they observed that the addition of 2.5 mg of potassium cyanide per cc of blood caused a decolorization of 2-6-dichlorophenolindophenol, corresponding to an ascorbic acid concentration of approximately 0.1 mg %. They were unable to explain the fact that the addition of the same amount of potassium cyanide to bloods of very high ascorbic acid content caused a slight decrease in the apparent ascorbic acid concentration. They concluded that there was no reason for the addition of potassium cyanide to blood for the determination of plasma ascorbic acid. Farmer and Abt[11] in studying the effect of potassium cyanide on the decolorization of 2-6-dichlorophenolindophenol concluded that its use invalidated plasma ascorbic acid values. Furthermore, they observed that during a 2-hour period there was a gradual and variable fall in the plasma ascorbic acid values when whole blood, plasma, or plasma filtrate stood at room temperature both in the presence and in the absence of cyanide.

This paper presents further evidence concerning the effect of potassium cyanide on 2-6-dichlorophenolindophenol, the stability of reduced ascorbic acid in whole blood, plasma, and plasma filtrate, and the question of whether potassium cyanide should or should not be used in plasma ascorbic acid determinations.

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[4] Kellie, A. E., and Zilva, S. S., *Biochem. J.*, 1935, **29**, 1028.

[5] Barron, E. S. G., de Meio, R. H., Klemperer, F., *J. Biol. Chem.*, 1936, **112**, 625.

[6] Pijoan, M., Townsend, S. R., and Wilson, A., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 224.

[7] Pijoan, M., and Klemperer, F., *J. Clin. Invest.*, 1937, **16**, 443.

[8] Pijoan, M., and Eddy, E., *J. Lab. and Clin. Med.*, 1937, **22**, 1227.

[9] Mindlin, R. L. M., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

[10] Friedman, G. J., Rubin, S. H., and Kees, W., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 358.

[11] Farmer, C., and Abt, A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 399.

*The effect of potassium cyanide on decolorization of 2-6-dichlorophenolindophenol in metaphosphoric acid solutions:* Table I shows that the decolorizing effect of potassium cyanide on the dye depends upon, one, the lot of potassium cyanide, two, the concentration of potassium cyanide, and three, the pH of the reaction mixture. The decolorizing effect was greater when the reaction mixture was pH 2, which is the pH of the titration procedures in common use, than at the pH of 4 which is used in the method of Mindlin and Butler.[9]

TABLE I.
Effect of Different Lots of KCN on Decolorization of 2-6-dichlorophenolindophenol in Metaphosphoric Acid Solutions Expressed as the Ascorbic Acid Equivalent in mg %, Had the Determinations Represented Plasma Analyses.

| Lot No. KCN | Mg KCN in sample analyzed | decolorization as mg % ascorbic acid | |
|---|---|---|---|
| | | pH 2 | pH 4 |
| M 1 | 0.6 | 0 | 0 |
| ,, | 2.0 | 0 | 0 |
| ,, | 4.0 | 0.2 | 0.1 |
| P 1 | 0.6 | 0.15 | 0.15 |
| ,, | 2.0 | 0.55 | 0.40 |
| ,, | 4.0 | 1.00 | 0.60 |

Recrystallization of potassium cyanide from a saturated water solution with or without the addition of alcohol has enhanced rather than reduced the decolorizing property of a poor lot of the salt. Precipitation of cyanide from a solution of the salt by the addition of a slight excess of silver nitrate and filtration gave a solution that did not decolorize the dye. Acidification of a solution of potassium cyanide with $H_2SO_4$, evaporation to dryness at room temperature, and addition of water to the original volume gave a solution whose decolorizing power was approximately the same as that of the original potassium cyanide solution.

Table II shows the importance of the 3 factors mentioned above on the effect of potassium cyanide in the determination of solutions of known ascorbic acid concentration.

Table III shows the range of error introduced into the determination of plasma ascorbic acid when the 2 brands of potassium cyanide referred to in Tables I and II are used at the pH 2 and 4. The data show that the use of cyanide, in the amounts recommended by Pijoan and Klemperer[7] and Mindlin and Butler,[9] in plasma ascorbic acid determinations does not necessarily invalidate them. Depend-

TABLE II.
Effect of Different Lots of KCN on Apparent Ascorbic Acid Concentration of a 1 mg % Ascorbic Acid Solution at pH 2 and pH 4.

| | | pH 2 | | | | pH 4 | |
|---|---|---|---|---|---|---|---|
| | | Titration[9] | | Photoelectric Col. | | Photoelectric Col.[8] | |
| Lot No. KCN | Mg KCN in sample analysed | mg% found | % error | mg% found | % error | mg% found | % error |
| | 0 | 1.0 | 0 | 1.0 | 0 | 1.0 | 0 |
| M 1 | 0.6 | 1.0 | 0 | 1.0 | 0 | 1.0 | 0 |
| " | 2.0 | 1.1 | + 10 | 1.0 | 0 | 1.0 | 0 |
| " | 4.0 | 1.2 | + 20 | 1.1 | +10 | 1.1 | +10 |
| P 1 | 0.6 | 1.1 | + 10 | 1.1 | +10 | 1.1 | +10 |
| " | 2.0 | 1.5 | + 50 | 1.4 | +40 | 1.3 | +30 |
| " | 4.0 | 2.0 | +100 | 1.8 | +80 | 1.4 | +40 |

ing upon the lot of cyanide there may be no error whatever, or an error amounting to 0.1 mg % in the case of the Mindlin and Butler[9] procedure, and 0.1-0.2 mg % in the case of the titration procedure.

TABLE III.
Effect of Different Lots of KCN upon Determination of Plasma Ascorbic Acid. Measurements Were Made by Means of Photoelectric Cell at pH 2 and 4.

| | Lot No. KCN | Mg KCN in sample analyzed | Mg% ascorbic acid | |
|---|---|---|---|---|
| | | | pH 2 | pH 4 |
| Plasma 1 | 0 | 0 | 0.6 | 0.6 |
| | P 1 | 0.6 | 0.8 | 0.7 |
| | M 1 | 0.6 | 0.6 | 0.6 |
| " 2 | 0 | 0 | | 0.9 |
| | P 1 | 1.0 | | 1.0 |
| " 3 | 0 | 0 | 0.5 | 0.5 |
| | P 1 | 0.6 | 0.6 | 0.6 |
| | P 1 | 1.0 | 0.8 | 0.7 |

This is an error which is not of clinical significance in a method in which the error is plus or minus 0.1 mg %. In our hands the limit of error of the method in which 2 cc of plasma is used is 0.2 mg % when the pH is less than 2.5, and 0.1 mg % when the pH is between 3.5 and 4.2. As shown by Mindlin and Butler[9] the greater experimental error at the more acid pH is due to the unequal rates of fading in the blank-dye solution and in the plasma-dye solution. When the pH of the final solution is between 3.5 and 4.2 this difference in the rate of fading is almost eliminated.

*The stability of reduced ascorbic acid in whole blood, plasma, or metaphosphoric acid plasma filtrate at room temperature with and* *without potassium cyanide.* Table IV shows the mg % of plasma ascorbic acid found after samples of whole blood, plasma, or metaphosphoric acid plasma filtrate stood for specified time intervals. In those samples to which potassium cyanide was added, 0.6 mg of potassium cyanide (lot M 1) was added per cc of whole blood. The data show that whole blood and metaphosphoric acid plasma filtrates stood as long as 24 hours without significant change in ascorbic acid concentration. When plasma stood at room temperature for over 4 hours, there was a significant progressive fall in ascorbic acid concentration. Although the different effects of standing observed by others[6, 11] may have been due to the presence of a catalyst, it is interesting to note that we made no particular attempt to eliminate such substances. The data also show that although the addition of potassium cyanide did not alter the observed concentrations of ascorbic acid, it did not prevent the progressive loss from the samples that stood as plasma.

TABLE IV.
**Effect of Time of Standing at Room Temperature of Whole Blood, Plasma, or Metaphosphoric Acid Plasma Filtrates with or without KCN on Plasma Ascorbic Acid Concentration. Results are given as mg% of plasma ascorbic acid.**

| Blood No. | Sample standing | Time of standing in hr | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 24† |
| 4 | Whole blood | 1.9 | 1.8 | 1.8 | | |
| | Plasma | 1.9 | 1.8 | 1.7 | | |
| | ” + KCN* | 1.8 | 1.7 | 1.7 | | |
| | Filtrate | 1.9 | 1.9 | 1.9 | | |
| 5 | Whole blood | 1.8 | | 1.8 | 1.8 | 1.7 |
| | ” ” + KCN* | | | | 1.7 | 1.6 |
| | Plasma | 1.8 | | | 1.5 | 1.2 |
| | ” + KCN* | 1.7 | | | 1.5 | 1.1 |
| | Filtrate | 1.8 | | | 1.8 | 1.7 |

*0.6 mg KCN lot M 1 per cc whole blood.
†First 6 hours at room temperature, last 18 hours in ice box.

*Conclusions.* The use of potassium cyanide as recommended in plasma ascorbic acid analyses[7, 9] does not necessarily invalidate the results and may cause no error. Certain lots of potassium cyanide may decolorize 2-6-dichlorophenolindophenol, the decolorizing power of a particular lot depending upon the concentration of the salt and the pH of the dye-salt solution. Reduced ascorbic acid is stable in whole blood or metaphosphoric acid plasma filtrates for as long as 24 hours. There is a significant loss of reduced ascorbic acid when plasma stands at room temperature for more than 4 hours. This loss is not prevented by the presence of potassium cyanide in concentrations recommended in plasma ascorbic acid analysis. These observations indicate that there is no reason for the addition of potassium cyanide in the determination of plasma ascorbic acid by the methods mentioned.

### 10264

## Immunological Differentiation of Shigella Alkalescens from *Shigella paradysenteriae* Flexner, V, W, X, Y, and Z.

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When Andrewes[1] described for the first time a new member of the Shigella group that is referred to today as *Shigella alkalescens,* his main purpose was to differentiate this microörganism from true dysentery bacilli. The purpose of this differentiation still holds true, since *Shigella alkalescens* does not cause epidemic or endemic forms of bacillary dysentery in man as does the closely related *Shigella paradysenteriæ* Flexner. Both microörganisms show similarities, not only in cultural characters but also in antigenic structure: *Shigella alkalescens* may be agglutinated by anti-Flexner sera and anti-*Shigella alkalescens* sera may agglutinate certain strains of *Shigella paradysenteriæ* Flexner as demonstrated by Andrewes,[1] Smith and Fraser,[2] Popoff and Spanswick,[3] Welch and Mickle,[4] Gilbert and Coleman,[5] Neter and Rappole,[6] and Neter.[7, 8] Recently, it was found that type-specific anti-*Shigella paradysenteriæ* Flexner sera differ markedly in their content of cross-agglutinins for *Shigella alkalescens.* While anti-*Shigella paradysenteriæ* Y sera strongly agglutinated *Shigella alkalescens,* and anti-*Shigella paradysenteriæ* V and X sera contained a moderate titer of agglutinins for this microörganism, anti-*Shigella paradysenteriæ* W and Z sera may lack agglutinins for *Shigella alkalescens* in spite of a high titer of agglutinins for the respective, homologous types of *Shigella paradysenteriæ* Flexner. It could be shown that certain strains of *Shigella paradysenteriæ* Flexner absorbed their cross-agglutinins from anti-*Shigella alkalescens* sera, and that such absorbed sera still agglutinated *Shigella alkalescens*. It is well known that it is not possible to absorb completely anti-*Shigella paradysenteriæ* Flexner sera with heterologous types of the Flexner group. The possibility has to be considered, that *Shigella alkalescens* may contain several antigenic components in common with certain types of the Flexner group and that the absorption of anti-*Shigella alkalescens* serum with only one type of the Flexner group may not remove all anti-Flexner agglutinins. It was decided, therefore, to absorb anti-*Shigella alkalescens* sera with all 5 Andrewes-Inman types, V, W, X, Y, and Z of the Flexner group in order to determine whether *Shigella alkalescens* contains an antigen characteristic of itself, which differentiates it immunologically from *Shigella paradysenteriæ,* V, W, X, Y, and Z of the Flexner group.

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[1] Andrewes, F. W., *Lancet,* 1918, **194**, 560.

[2] Smith, J., and Fraser, A. M., *J. Path. and Bact.,* 1928, **31**, 511.

[3] Popoff, N. W., and Spanswick, M. P., *J. Lab. and Clin. Med.,* 1931, **16**, 437.

[4] Welch, H., and Mickle, F. L., *Am. J. Publ. Health,* 1934, **24**, 219.

[5] Gilbert, R., and Coleman, M. D., *Am. J. Publ. Health,* 1934, **24**, 449.

[6] Neter, E., and Rappole, F., *Arch. Path.,* 1938, **25**, 298.

[7] Neter, E., *J. Bact.,* 1938, **35**, 202.

[8] Neter, E., *J. Immunology,* 1938, **35**, 339.

The experiment was set up as follows: Anti-*Shigella alkalescens* serum, in a dilution of 1:25 was mixed with the sediment of heavy suspensions of (a) *Shigella paradysenteriæ* V; (b) *Shigella paradysenteriæ* W; (c) *Shigella paradysenteriæ* X; (d) *Shigella paradysenteriæ* Y; (e) *Shigella paradysenteriæ* Z; (f) a mixture of *Shigella paradysenteriæ* V, W, X, Y, and Z, and (g) *Shigella alkalescens*. The sediment was suspended in the serum and the mixtures were kept in the ice chest for 3 hours. Frequently the sediment was stirred up. Then the tubes were centrifuged at high speed for one hour, until the supernate was clear. The supernates, as well as the non-absorbed native serum dilution were mixed with the 5 types of *Shigella paradysenteriæ* V, W, X, Y, and Z as well as with a suspension of *Shigella alkalescens*. The tubes were incubated in a water bath at 55°C for 24 hours. The resulting agglutination was read macroscopically and noted at various intervals. The result of this experiment is recorded in Table I.

It may be seen from Table I that the native anti-*Shigella alkalescens* serum agglutinated *Shigella alkalescens;* it also caused agglutination of *Shigella paradysenteriæ* V, X, and Y. After absorption of the serum with *Shigella alkalescens,* no agglutination occurred with any of the strains tested. The table, furthermore, shows that after absorption with the suspension of one type of *Shigella paradysenteriæ* Flexner, the agglutinins for this type were completely removed; such an absorbed serum, however, still agglutinated more or less other types of the *Shigella paradysenteriæ* group. It is evident from Table I that after absorption of the anti-*Shigella alka-*

TABLE I.
Agglutination of *Shigella paradysenteriæ* V, W, X, Y, Z and *Shigella alkalescens* by Native and Absorbed Anti-*Shigella alkalescens* Serum.

| Anti-*Shigella alkalescens* Serum. | *S. paradysenteriæ* | | | | | *S. alkalescens* |
|---|---|---|---|---|---|---|
| | V | W | X | Y | Z | |
| I<br>Native | + | — | +++ | +++ | — | ++++ |
| II<br>Absorbed with *S. alkalescens* | — | — | — | — | — | — |
| III<br>Absorbed with *S. paradys.* V | — | — | — | ++ | — | ++++ |
| IV<br>Absorbed with *S. paradys.* W | + | — | + | ++ | — | ++++ |
| V<br>Absorbed with *S. paradys.* X | + | — | — | ++ | — | ++++ |
| VI<br>Absorbed with *S. paradys.* Y | ++ | — | — | — | — | ++++ |
| VII<br>Absorbed with *S. paradys.* Z | + | — | + | ++ | — | ++++ |
| VIII<br>Absorbed with *S. paradys.* V, W, X, Y, Z | — | — | — | — | — | ++++ |

— = No agglutination.
+, ++, +++, ++++ = Increasing degrees of agglutination.

*lescens* serum with any of the 5 types, V, W, X, Y, and Z of *Shigella paradysenteriæ* Flexner, the absorbed serum still agglutinated *Shigella alkalescens* and—as quantitative titrations revealed—to about the same titer as the native, non-absorbed serum. When the anti-*Shigella alkalescens* serum was absorbed with a mixture of the 5 types, V, W, X, Y, and Z of *Shigella paradysenteriæ,* all the agglutinins for *Shigella paradysenteriæ* V, W, X, Y, and Z were removed and yet the serum still agglutinated *Shigella alkalescens* without any demonstrable loss in its titer. Essentially the same results were obtained in several experiments, employing 3 different anti-*Shigella alkalescens* sera.

In view of the observations of Boyd[9] and others, that the Flexner group comprises additional types, which are antigenically different from the Andrewes-Inman types, V, W, X, Y, and Z, further experiments are necessary in order to determine whether or not antigenic relations exist between *Shigella alkalescens* and the remaining types of the *Shigella paradysenteriæ* Flexner group.

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[9] Boyd, J. S. K., *J. Hyg.,* 1938, **38,** 477.

In conclusion, *Shigella alkalescens* contains in addition to antigenic components in common with *Shigella paradysenteriæ* Flexner, an antigen that is not shared by the Andrewes-Inman types V, W, X, Y, and Z of *Shigella paradysenteriæ* Flexner and that apparently is characteristic of itself.

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### 10265 P

## Functional Activity of Pancreatic Ampulla in Rabbit.

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In rabbit, the pancreatic ampulla is entirely separate from the biliary ampulla and its functional activity has not been described as far as we know; moreover both ampullae may be studied at the same time. This structure is located about 15 cm distal to the bile ampulla and varies between 5 x 3 and 7 x 3 mm. After exposure under sodium barbital narcosis little or no activity is generally seen. Intravenous injection of a crude "secretin" mixture, not assayed for histamin, in 0.5 cc per kg doses brings about a series of events in the pancreatic ampulla that are similar in general to observations made on the biliary ampulla by Westphal in guinea pig, rabbit and cat[1] and by Burget and Brocklehurst in guinea pig.[2] After a latent period of 15-30 seconds, a contraction less than 0.5 mm in width appears at the junction of the pancreatic ampulla and duct. This blanched area then relaxes, but the contractions reappear with increased frequency and increased strength, often involving more of the duct, until finally a peristaltic wave of contraction beginning at the duct sweeps over the entire ampulla changing it into a grayish-white, linear, puckered mass. Such a complete contraction may persist for 1-2 seconds and then relaxation sets in. The relaxation betrays itself as a pink flush that generally proceeds from the distal end of the ampulla to its junction with the duct; occasionally the relaxation apparently occurs at the same time throughout the contracted area. The number of separate contractions and relaxations may reach 10 per minute but only a fraction are complete. The contractions persist in decreasing number for about 10 minutes; occasionally for more than 20 minutes.

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[1] Westphal, K., *Z. f. Klin. Med.*, 1923, **96**, 52.

[2] Burget, G. E., and Brocklehurst, R. J., *Am. J. Physiol.*, 1927-28, **83**, 578.

The adjacent duodenum may or may not follow an ampullar contraction with a contraction of its own. Frequently either duodenum or ampulla may contract without involving the other.

After slitting the duodenum (cautery) the pancreatic ampulla is seen as a grayish-pink knob. With a complete contraction, this knob becomes slightly more prominent, an oval slit appears in its proximal side and a small amount of clear fluid is expelled with more or less abruptness. During issue of this juice the knob pales and becomes less prominent; the opening disappears after expulsion of the secretion.

The same course of events is seen in the *bile* papilla of rabbit. Relaxation is observable with greater ease because the bile papilla is larger. The relaxation generally proceeds as a pink blush from the distal end of the papilla to the choledochoduodenal junction; in the same animal, however, the relaxation may at times begin at both ends of the papilla, or the relaxation travels, like the contraction wave, from the choledochus junction to the tip of the papilla.

## 10266

## Degree of Immunological Specificity of the Nondialysable Growth Products of *Eberthella, Salmonella, Brucella,* and *Proteus.*

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Morell and Shwartzman[1] recently reported the successful use of dialysis in the preparation and purification of certain immunologically active bacterial products, particularly those of the meningococci. From their work it would appear that nondialysates of this kind carry a rather high degree of specificity. It was with the idea of improving upon the specificity of the Weil-Felix reaction that the work here reported was begun. Using their methods, we were able to secure immunologically reactive products from proteose-peptone cultures of *Eberthella typhosa, Salmonella paratyphi, Salmonella schottmulleri,* and *Proteus X19* (0504 strain) which flocculated with homologous immune (rabbit) serum. Four days were allowed for culturing and 4 days for dialysis in each instance. Serum from proven cases of endemic typhus cases also flocculated with the *Proteus* non-dialysate. Tests were carried out by layering dilutions of the nondialysate over whole serum. In the positive reactions a visible flocculate formed within 30 minutes at room temperature, about 23°C. Agglutination tests with the same bacterial antigens were carried out at the same time.

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[1] Morell, S., and Shwartzman, G., *Science*, 1937, **86**, 130.

TABLE I.

**Flocculation Between Endemic Typhus Serum and the Nondialysable Growth Products of *Proteus X19, E. typhosa, S. paratyphi,* and *Br. abortus.* Agglutination of the same organisms by these samples of serum is shown in the adjoining column.**

| Typhus Serum | *Proteus X19* | | *E. typhosa* | | *S. paratyphi* | | *Br. abortus* | |
|---|---|---|---|---|---|---|---|---|
| | Flocc. | Agglu. | Flocc. | Agglu. | Flocc. | Agglu. | Flocc. | Agglu. |
| 1 | 1:256 | 1:1280 | 1:4 | 1:40 | 0 | 1:20 | 0 | 1:20 |
| 2 | 1:256 | 1:1280 | 1:4 | 1:30 | 0 | 0 | 0 | 1:40 |
| 3 | 1:128 | 1:640 | 0 | 1:20 | 0 | 0 | 0 | 1:30 |
| 4 | 1:64 | 1:240 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 1:128 | 1:240 | 1:4 | 1:30 | 0 | 0 | 1:4 | 1:40 |
| 6 | 1:512 | 1:1280 | 1:8 | 1:120 | 1:4 | 1:20 | 1:16 | 1:80 |
| 7 | 1:128 | 1:480 | 1:8 | 1:80 | 0 | 0 | 1:4 | 1:40 |
| 8 | 1:128 | 1:480 | 1:4 | 1:60 | 0 | 0 | 1:4 | 1:40 |

The results (Table I) show that typhus serum flocculated with the nondialysates of *Proteus X19* at reasonably high titers, at the same time failing to do so at similarly high titers with the nondialysates of *E. typhosa, S. paratyphi,* and *S. schottmulleri.* The agglutinations here recorded of bacterial suspensions by the same serum are not out of keeping with the established relationships between the organisms used. Since the Weil-Felix reaction is still considered as one of the heterophile phenomena, it would scarcely be expected that this method would discover a strict degree of spec-

TABLE II.

**Flocculation Between the Nondialysable Growth Products of *Proteus X19, E. typhosa, S. paratyphi,* and *Br. abortus* and Immune Sera. Agglutination is shown in the adjoining column.**

| Immune Sera | *Proteus X19* | | *E. typhosa* | | *S. paratyphi* | | *Br. abortus* | |
|---|---|---|---|---|---|---|---|---|
| | Flocc. | Agglu. | Flocc. | Agglu. | Flocc. | Agglu. | Flocc. | Agglu. |
| *Proteus X19* | 1:512 | 1:5120 | 1:64 | 1:240 | 1:4 | 1:40 | 1:32 | 1:240 |
| *E. typhosa* | 1:32 | 1:160 | 1:512 | 1:6400 | 1:64 | 1:480 | 1:32 | 1:240 |
| *S. paratyphi* | 1:8 | 1:40 | 1:32 | 1:320 | 1:512 | 1:6400 | 1:8 | 1:60 |
| *Br. abortus* | 1:8 | 1:40 | 1:8 | 1:120 | 1:4 | 1:40 | 1:256 | 1:5120 |

ificity between the nondialysates and the immune substances present in typhus serum. Still, the flocculation tests seemed to exhibit more specificity than was found in agglutination tests with typhus serum. Such a degree of specificity was not found in the flocculations between the nondialysates of the other cultures here used and the other immune sera. Instead, the results paralleled the behavior of agglutinating reactions between these immune sera and related organisms (Table II). Under these conditions and with these organisms, flocculation with nondialysates and immune serum seem to be no more specific than the group agglutinations commonly observed between the species used. It seems reasonable to conclude, therefore, that the *in vitro* reactions of the nondialysable growth products of microorganisms studied are without a strict degree of specificity.

### 10267

## Vitamin-C Inhibition of Agglutinin Production.

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In a previous paper it was shown that ascorbic acid added to or injected simultaneously with horse serum hastens and augments specific-precipitin production in rabbits. A 10- to 30-fold increase in precipitin-titer is readily produced by optimal doses (about 50 mg) of this enzyme-activator. We have extended the study of the potentiating action of ascorbic acid to other antigens. The present paper summarizes our experimental data with bacterial vaccines and sheep erythrocytes (Forssmann antigen).

About 200 two kg rabbits were divided into groups of 6. Each group was injected intravenously with an arbitrary dose of sheep erythrocytes, or of living, heat-killed or formalin-killed suspensions of *B. proteus*, *B. coli* or *B. typhosus*. One minute after each injection, 3 members of each group were given an intravenous injection with 50 mg ascorbic acid dissolved in 1 cc NaCl-solution.

Very low antibody-titers were obtained as a result of these single injections. Within the limits of the experimental error ascorbic acid had only border-line effects on antibody-yield, except on the yield of specific agglutinin. In 3 groups injected with *B. typhosus,* for example, the yield of specific agglutinins was reduced about 50%.

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In order to magnify these border-line effects, additional groups of 6 rabbits were given from 7 to 13 multiple injections with the same antigens, followed in half of the members of each group by our routine therapeutic dose (50 mg) of ascorbic acid. In addition to the erythrocytes and bacterial vaccines above tested, 3 groups of rabbits were injected with partially autolysed or 'phage-lysed *B. typhosus* and 2 groups with staphylococcal proteins obtained by grinding staphylococci in a ball-mill. For the staphylococcal proteins used in these tests we are indebted to Dr. A. P. Krueger, of the University of California. Typical data from these multiple injections are recorded in Table I.

TABLE I.

Multiple Injections of 'Phage-lysed *B. typhosus* and Ascorbic Acid.

Intravenous injections with 1 cc of a 10-hour 'phage-lysed *B. typhosus* culture at 3- to 5-day intervals, followed immediately with 50 mg ascorbic acid also given intravenously. Half of the members of each group (controls) were given no ascorbic acid. Bleedings were made at irregular intervals, usually at least 5 days after the previous injection. (Number of previous injections shown by the Roman numerals.) The resulting antiserums were titrated for specific precipitins (ring test) and specific agglutinins. The low precipitin-titers recorded are due to the use of a very dilute Berkefeld-filtrate of 'phage-lysed *B. typhosus* as the test-antigen. The recorded precipitin-titers should be multiplied by at least 1000 to make them quantitatively comparable with the specific agglutinin titers.

Almost identical average titers were obtained in the 2 series of rabbits injected with staphylococcal proteins.

| | 25th day VI | 45th day XI | 53d day XII | 60th day XIII | 67th day XIII | 74th day XIII | Avg % |
|---|---|---|---|---|---|---|---|
| Precipitin-titer | | | | | | | |
| Ascorbic acid group | 0 | 1:4 | 1:9 | 1:8 | 1:10 | 1:5 | 130 |
| Control group | 0 | 1:3 | 1:6 | 1:7 | 1:8 | 1:4 | 100 |
| Agglutinin-titer | | | | | | | |
| Ascorbic acid group | 0 | 1:1800 | 1:4500 | 1:1500 | 1:1000 | 1:500 | 40 |
| Control group | 0 | 1:4700 | 1:7500 | 1:6400 | 1:2500 | 1:1500 | 100 |

From this table it is seen that multiple doses of ascorbic acid increase specific-precipitin production against bacterial vaccines about 30%. This increase, however, is negligible when compared with the 10- to 30-fold increase previously obtained with horse serum. This border-line beneficial effect, however, is more than offset by the 60% reduction in the average yield of specific agglutinins.

While ascorbic acid is thus without apparent clinical promise as a potentiator of bacterial vaccines, the above dual effect is of basic theoretical interest. The slight but distinct increase in specific-precipitin production coupled with a marked decrease in the yield of specific agglutinins, is conclusive evidence of the existence of at least 2 different physiological mechanisms for specific-antibody production.

A plausible explanation would be furnished by the hypothesis that there are 2 competitive defensive mechanisms operative in humoral immunity: (i) an extracellular synthesizing process mainly operative against relatively non-toxic alien proteins and responsible for specific-precipitin production, and (ii) an intracellular lytic process mainly operative against phagocyted microörganisms or microbic fragments. Vitamin-C activation of tissue enzymes, therefore, might conceivably increase precipitin-production while reducing the yield of specific agglutinins by causing a more rapid intracellular destruction of phagocyted antigens.

If this is true, vitamin C therapy would have a predictable clinical value in the treatment of specific infectious diseases, even though it is of no apparent clinical promise as an adjuvant in vaccine therapy. Recent reported successes of vitamin C therapy in the treatment of experimental poliomyelitis and tuberculosis are in line with this prediction.

## 10268

## Cerebrospinal Pressure, Hydrocephalus and Blood Pressure in the Cat Following Intracisternal Injection of Colloidal Kaolin.

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Certain animals develop an increased intracranial pressure associated with vascular hypertension following the intracisternal injection of colloidal kaolin. This syndrome was first described in the dog by Heller[1] and his associates, and recently confirmed by Jeffers, Lindauer and Lukens.[2] A similar response in the white rat has been described by Griffith, Jeffers, and Lindauer,[3, 4] and Griffith and Roberts.[5] They found that the cerebrospinal pressure, normally less that 100 mm of water, was increased to about 260 mm, while the blood pressure, normally below 140 mm of mercury, ranged between 150 and 300 mm. At necropsy internal hydrocephalus was found in rats that had survived a month or more. The present report deals with similar studies in cats.

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* Atwater Kent Fellow in Medicine.

[1] Dixon, W. E., and Heller, H., *Arch. f. exp. path. u. pharm.*, 1932, **166**, 265.

[2] Jeffers, W. A., Lindauer, M. A., and Lukens, F. D. W., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 260.

The method for measuring blood pressure was that described by Griffith and Collins[6] for man and by Griffith[7] for the rat. It consists in encircling the upper part of a limb with a blood pressure cuff constructed of appropriate size, while blood flow is observed through the microscope in a more distal cutaneous area of this same limb. This area usually has to be prepared in advance by shaving. When the pressure in the cuff exceeds systolic pressure the flow in the capillaries stops, to start again when the pressure in the cuff is lowered below systolic pressure. Thus systolic pressure alone is measured.

Blood pressure measurements were made on about 24 normal cats. Ether anesthesia was used throughout. Cisternal puncture was successfully performed on 15 cats. In approximately half of these the pressure of cerebrospinal fluid was measured directly by permitting the fluid to pass into a graduated capillary tube, so narrow that the loss of fluid was only 0.2 cc per 100 mm rise. Then 0.5 cc of cerebrospinal fluid was withdrawn, and a kaolin suspension slightly less in volume was injected. The kaolin suspension consisted of 25% by volume of kaolin boiled up in distilled water and permitted to cool before injecting.

These 15 cats had repeated blood pressure measurements at approximately weekly intervals. Three animals had measurements of cerebrospinal pressure made 3-5 weeks after the injection of kaolin. Six animals were killed and formalin injected into the internal carotid artery immediately after death. The brain was thus fixed *in situ* and subsequently removed.

In the normal cat under ether anesthesia the systolic blood pressure by this method ranges between 85 and 130 mm of mercury, while the cerebrospinal pressure does not exceed 100 mm of water.

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[3] Griffith, J. Q., Jr., Jeffers, W. A., and Lindauer, M. A., *Am. J. Physiol.*, 1935, **113**, 285.

[4] Griffith, J. Q., Jr., Jeffers, W. A., and Lindauer, M. A., *Am. J. Physiol.*, 1937, **118**, 1.

[5] Griffith, J. Q., Jr., and Roberts, E., *Am. J. Physiol.*, 1938, **124**, 86.

[6] Griffith, J. Q., Jr., and Collins, L. H., Jr., *Am. Heart J.*, 1922, **8**, 671.

[7] Griffith, J. Q., Jr., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 394.

Following the injection of kaolin into the cistern the blood pressure, as a rule, remained within normal limits. Three readings were obtained ranging between 155 and 165 mm on different animals, but this trifling hypertension had returned to normal by the next week in 2 animals, while the third died before a subsequent measurement had been made.

The cerebrospinal pressure was definitely increased, being 140, 158, and 175 mm of water in 3 animals with consistently normal blood pressures. All animals autopsied showed marked internal hydrocephalus, this being present 4 or more weeks after the injection of kaolin.

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### 10269

## Enzymes in Orthopteran Ontogenesis. VI. Autocatalytic Nature of *in vivo* Formation of Protyrosinase.*

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*From the Zoological Laboratory, State University of Iowa.*

Northrop[1] considered the autocatalytic formation of pepsin and trypsin from their zymogens as being an instance of protein synthesis. Therefore, since Wrinch's[2] theory described strata of two-dimensional, cyclol-structured lamina, Langmuir and Schaefer[3] point out that protein growth, as with crystal formation, is a determined one and should reasonably be autocatalytic. In the light of these observations, it should be of some general interest to describe the *in vivo* formation of an enzyme precursor. The present paper is concerned with the reporting of the autocatalytic nature of the formation of protyrosinase within eggs of the grasshopper, *Melanoplus differentialis*.

The preparation and activation of grasshopper egg protyrosinase has been dealt with in a series of papers (Bodine and Boell,[4] Bodine, Allen, and Boell,[5] Bodine and Allen[6, 7]) so that here a brief statement of the essential procedures should suffice. Centrifugation of an egg brei prepared in a physiological normal, phosphate buffered (pH 6.8) sodium chloride solution results in the formation of 3 layers, termed A, B, and C respectively, from the centripetal to the centrifugal poles. The lipoidal layer A contains a naturally occurring activator of the protyrosinase which occurs entirely in the cell-free, comparatively voluminous B layer. Layer C consists of the inert egg membranes and structural elements. The brei was always diluted to such a volume that this precursor or inactive form of tyrosinase present in 1.0 cc of fraction B was that derived from 20 eggs irrespective of their particular age.

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* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[1] Northrop, J. H., *Physiol. Rev.*, 1937, **17**, 144.

[2] Wrinch, D. M., *Roy. Soc.*, 1937, A **160**, 59.

[3] Langmuir, I., and Schaefer, U. J., *J. Am. Chem. Soc.*, 1938, **60**, 1351.

[4] Bodine, J. H., and Boell, E. J., *J. Cell. and Comp. Physiol.*, 1935, **6**, 263.

[5] Bodine, J. H., Allen, T. H., and Boell, E. J., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 450.

[6] Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938a, **11**, 409.

[7] Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938b, **12**, 71.

A solution of tyramine hydrochloride was used for substrate in such an amount that during the tyrosinase-catalyzed oxidation to the end product, melanin, 388 mm$^3$ of oxygen was consumed. The rate of this oxygen uptake was determined in the standard Warburg manometer at 25°C and was expressed as the reciprocal of the time necessary for half completion of the reaction.

As previously pointed out, sodium oleate appears to be an "ideal" activator in the sense that once superoptimal amounts of this compound are present, all the protyrosinase presumably becomes tyrosinase (Bodine and Allen[7]). In many instances it is a general rule that the rate of enzymatically catalyzed reactions varies directly with the amount of enzyme (Tauber[8]). Such has been found to be true (Bodine and Allen[9]) for the amounts of tyrosinase used in the present experiment. Therefore, it seems proper to consider the rate of oxidation of tyramine hydrochloride as a measure of the quantity of tyrosinase. In turn, these rates may actually be interpreted as a comparison of the various amounts of protyrosinase, since as mentioned above this inactive form is converted quantitatively to the active one by the sodium oleate.

A convenient form of the simple autocatalytic reaction (Herriott[10]) may be stated

$$+\frac{dA}{dt} = KA(A_e—A)$$

which after integration is

$$K = \frac{2.303}{A_e t} \log \frac{A}{A_e—A} \frac{A_e—A_o}{A_o}$$

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[8] Tauber, H., *Enzyme Chemistry,* 1937, John Wiley and Sons.

[9] Bodine, J. H., and Allen, T. H., unpublished observations, 1937.

[10] Herriott, R. M., *J. Gen. Physiol.,* 1938, **21**, 501.

where A is the rate at time t and $A_e$ is the equilibrium and $A_0$ the initial value of A. In Table I, K is calculated as derived by the equation from the mean value for the amount of protyrosinase obtained from 20 eggs of t days of age. Actually the eggs must be 8 days of age before an initial determination of protyrosinase may be made (Bodine, Allen, and Boell[5]). Therefore, t + 7 days of chronological age, *i. e.*, days' development at 25°C, is t days of the equation. The mean value of K, 0.00138, has been used in the construction of the theoretical curve (Fig. 1). Inspection reveals that this falls reasonably close to the probable errors of the means (Table I).

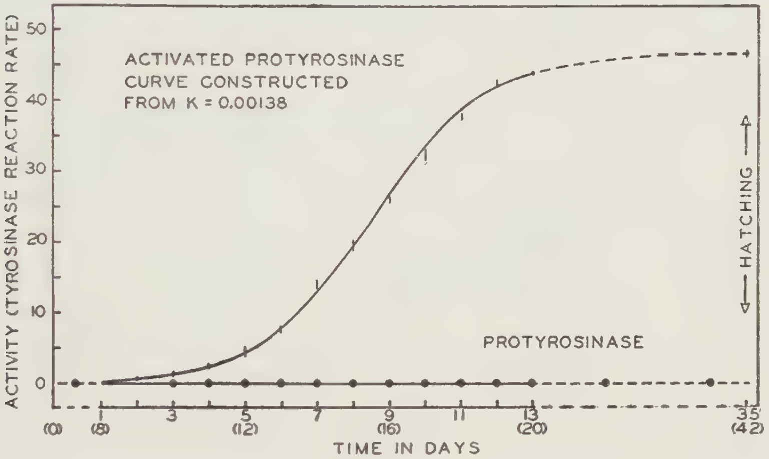

Fig. 1.

Formation of protyrosinase during development of the grasshopper egg. Ordinate denotes reciprocal of half oxidation period of tyramine hydrochloride $\times 10^3$. First abscissa gives days of apparent increase in protyrosinase, that is, t of the autocatalytic equation. Second abscissa (in parentheses) states days of development at 25°C minus the diapause period. The vertical lines of the theoretical curve represent the probable error of the means for determinations of protyrosinase activated by a hypercritical amount of sodium oleate. The solid circles show the inactivity of protyrosinase, *i. e.*, protyrosinase in the absence of sodium oleate. For further description see text.

In the absence of any data from experimental manipulation, such as variation of the ratio of end product to reactant concentrations, it seems best to merely examine the significance of the autocatalytic equation. This equation may be derived from either of 2 assumptions as regards the introduction of an equation of constraint. Either the constraint may be introduced as a function of the concentration of an intermediate acting as a catalyst in the formation of the end product, or the catalytic influence may be attributed to the operation

TABLE I.

| Days development (25°C) | t (days) of equation | $A_e = 46.5$<br>$A_o = 0.2$<br>K | Rate of tyramine hydrochloride oxidation catalyzed by activated protyrosinase | |
|---|---|---|---|---|
| | | | Theoretical | Experimental |
| 8 | 1 | — | — | *0.2 ± .18 |
| 9 | 2 | .00136 | 0.7 | *0.7 ± .14 |
| 10 | 3 | .00137 | 1.3 | *1.3 ± .45 |
| 11 | 4 | .00139 | 2.5 | 2.5 ± .51 |
| 12 | 5 | .00138 | 4.5 | 4.5 ± .68 |
| 13 | 6 | .00138 | 7.8 | 7.8 ± .51 |
| 14 | 7 | .00142 | 12.8 | 14.2 ± .71 |
| 15 | 8 | .00141 | 19.5 | 19.6 ± .71 |
| 16 | 9 | .00136 | 26.8 | 26.1 ± .60 |
| 17 | 10 | .00134 | 33.6 | 32.1 ± .77 |
| 18 | 11 | .00134 | 38.6 | 37.4 ± .28 |
| 19 | 12 | .00139 | 42.0 | 42.4 ± .24 |
| 20 | 13 | .00137 | 43.9 | 44.0 ± .21 |
| 42 | 35 | — | — | 46.5 ± .22 |

*Rate determined by extrapolation; all other rates by interpolation.

of the end product itself. The resulting forms of the final differential equations are identical.†

The very nature of autocatalytic reactions (Northrop[1]) intimates that at least one unit of the catalyst must be present among the reactants for the reaction to take place. Perhaps, in the way of speculation, the action of some gene mechanism may not be adverse to these observations (Gulick[11]).

It seems logical to conclude that within the grasshopper egg a precursor of tyrosinase is formed by autocatalysis.

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### 10270 P

## Variation in Distribution of Type and Group Substances in Smooth-Phase Cultures of Group A Beta Hemolytic Streptococci.

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For a decade it has been recognized that the amount of type-substance in different cultures of the same serological type of Group

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† For these suggestions the authors are indebted to Dr. Gordon Marsh of this department.

[11] Gulick, A., *Quart. Rev. Biol.*, 1938, **13**, 140.

A hemolytic streptococci manifests considerable variation. The limits of this antigenic variability in different strains of the same type, in different culture-phases of the same strain or in different colony cultures of the same strain and phase have, however, never been clearly related to the chief dissociative variants, with the exception of the early reports of Todd and Lancefield[1] and of Lancefield and Todd[2] on smooth and matt forms, and the later observations of Griffith.[3]

Observations on dissociative behavior had suggested to us that, among the variability-phenomena often associated with the R to S "reversion", there might arise smooth strains in which the type- or group-factor had been largely lost. A detailed study of cultures of hemolytic streptococci undergoing the R to S transformation has shown marked quantitative differences relating to type- and group-substances in different smooth-colony lines derived from the rough form. Study of these type- and group-cultures was of special interest because they represented antigenic variants within a single culture-phase.

From a smooth Type 5 strain a stable rough form was produced by encouraging the development of rough, marginal "outbursts" on typical smooth colonies. Upon reversion of this rough to smooth, following rapid broth-passages, 2 smooth colonies, macroscopically identical and containing organisms of similar morphological, cultural, and biochemical characters, were selected for further investigation. One colony-culture, 10-1S, gave maximal reactions, by the slide-agglutination technic of Griffith,[3] in Group A serums but no reaction in Type 5 serums from which the group-antibodies had been removed by absorption with a heterologous-type strain. The other colony-culture, 10-2S, gave maximal reactions in Type 5 serums but no reaction in Group A serums. It thus appeared that the former culture was heavily endowed with *group*-substance while the latter appeared to be equally endowed with *type*-substance. These 2 cultures resembled, respectively, the "group-" and "type-specific" phases mentioned by Griffith.[3] The antigenic characteristics of each culture have remained unchanged for about one year.

The absence of type-substance in the group-specific (GS) strain was further demonstrated by the precipitin-test. This test also showed that the type-specific (TS) culture possessed a fraction of group-substance. Moreover, in reciprocal slide-agglutination tests between TS and GS an unabsorbed serum immune to TS agglutinated both itself and GS, while the unabsorbed serum immune to GS agglutinated only its homologous antigen. It thus appeared that there was sufficient group-substance in the TS culture to produce some group-antibodies, but not a sufficient amount to yield positive slide-agglutination with GS serum. Moreover, in the GS culture there was not sufficient type-substance to produce serums capable of giving type agglutination with TS 10-2S antigen. Our results suggested that slide-agglutination was more useful than precipitation for the identification of TS and GS strains.

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[1] Todd, E. W., and Lancefield, R., *J. Exp. Med.*, 1928, **48**, 751.

[2] Lancefield, R., and Todd, E. W., *J. Exp. Med.*, 1928, **48**, 769.

[3] Griffith, F., *J. Hyg.*, 1935, **34**, 542.

Additional evidence of the antigenic diversity of the TS and GS lines was furnished by absorption-tests. One sample of a crude Type 4 serum was absorbed with TS 10-2S antigen and a similar sample was absorbed with GS 10-1S. Absorption with TS removed no group-antibodies, while absorption with GS removed all group-antibodies capable of acting on stock cultures of Types 1 to 10 as well as on GS 10-1S itself.

The interesting question arose: Was the segregation of type- and group-substances in TS and GS cultures (arising from 2 sister-colonies and representing *the same culture-phase*) sufficiently great to make possible the complete removal of group-antibodies from the TS serum as a result of absorption with the GS antigen, and without at the same time reducing the type-specific-antibody content? Such "homologous absorption" has been postulated by Griffith[4] as the "ideal method" for the preparation (by absorption) of type-serums, although successful results have not, we believe, been reported.

A sample of TS 10-2S serum was absorbed with GS 10-1S culture. When this absorbed serum was tested by slide-agglutination

TABLE I.

Conservation of type and loss of group antibodies in a smooth Type 5 serum (TS 10-2S) absorbed with a group-specific, smooth variant of the same strain (GS 10-1S). Homologous type, strain and phase absorption. Slide agglutination technic.

| Antigens | Unabsorbed antiserums | | Type 5 antiserum absorbed† as indicated below | | | | Control Rabbit Serum |
|---|---|---|---|---|---|---|---|
| | 10-1 | 10-2 | Type 10 | Once* 10-1 | Twice 10-1 | Once 10-2 | |
| GS 10-1S | 4+ | 4+ | — | — | — | — | — |
| TS 10-2S | — | 4+ | 4+ | 4+ | 4+ | — | — |

* This sample of absorbed serum was divided into 2 fractions. One was absorbed again with 10-1S (Column 5). The other was absorbed with 10-2S (Column 6).

† Serum diluted 1:3 (3 cc) absorbed with sediment from 1500 cc of broth culture.

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[4] Griffith, F., *Proc. Second Internat. Cong. for Microbiol.*, London, 1936, p. 133.

it was found that the absorption had removed all group-antibodies capable of reacting with the GS 10-1S culture as well as with stock cultures of Types 1 to 10. This absorption had not, however, removed the specific Type 5 antibodies capable of reacting with the TS 10-2S culture. A second absorption with the GS culture left the type-antibody content still unchanged. These results, as shown in Table I, again demonstrated the absence of type-substance in the smooth variant, 10-1S.

These results appear to suggest that a detailed examination of smooth-colony variants of other serological types of Group A *beta* hemolytic streptococci, isolated upon reversion from the rough, might result in the discovery of highly type-specific and group-specific lines whose employment would facilitate preparation of type- and group-serums appropriate for identification of streptococcal cultures.

## 10271 P

## Permanent Diabetes Insipidus Possible in the Absence of the Pars Anterior.*

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von Hann,[1] on the basis of pathological material, suspected that a functional pars anterior of the hypophysis was essential for a deficiency in the antidiuretic principle to be evident in the form of d.i. This contention has been championed particularly by Richter[2] and Ingram and Fisher[3] on the basis of experimental material. The experiments reported below reinforce data already on record,[4] which demonstrate that a moderate d.i. can obtain in the total absence of the pars anterior.

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* Aided by a grant from the Rockefeller Foundation.

[1] von Hann, F., *Frankfurt. Z. f. Path.*, 1918, **21**, 337.

[2] Richter, C. P., *Am. J. Physiol.*, 1936, **117**, 46, and *A. R. N. M. D.*, 1938, **17**, 392.

[3] Ingram, W. R., and Fisher, C., *Anat. Rec.*, 1936, **66**, 271.

[4] (a) Richert, F. L., and Dandy, W. E., *Bull. Johns Hopkins Hosp.*, 1936, **58**, 418; 1925, **37**, 1; (b) Keller, A. D., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 31; *Am. J. Physiol.*, 1938, **123**, 111; (c) White, H. L., and Heinbecker, P., *Am. J. Physiol.*, 1938, **123**, 213.

The water exchange was followed in 8 adult dogs for 6 months or longer after total hypophysectomy. Total hypophysectomy was made certain at operation by deliberate encroachment upon the ventral hypothalamus and cauterization of the base of the pituitary fossa. The dogs were maintained on a constant daily food intake, 50 g of baloration per kilo of body weight per day. Water was available to them 22 hours of each 24. The food given and water drunk was calculated for the duration of the experiments on the basis of the operation weight, which ranged from 6 to 10 kilo.

Without exception, there was a decided increase in the water exchange following operation which persisted indefinitely (10 months). In some instances the increase was immediate with no subsequent tendency to a "latent period". In other instances the rise occurred progressively over a period of several days. Usually the polydipsia was maintained at a higher level during the immediate 2-3 weeks after operation than it was in the final stationary state (after 3 to 6 weeks). The magnitude of the polydipsia present in the final state varied among the animals from 150 to 350 cc per kilo per day, the mean being around 300 cc. The preoperative water intake was in the neighborhood of 50 cc per kilo per day. There was always a corresponding polyuria of like magnitude, the urine being sugar-free and of low specific gravity.

Post operative evidence of the absence of the pars anterior was a marked increase in insulin sensitivity (20x), an appreciable reduction in basal metabolism, and degenerative changes in the suprarenals and thyroids of the animals that have been sacrificed.

The maximal polydipsia encountered in this series in the final state is below the maximal encountered in dogs under the same regime, where d.i. was precipitated by hypothalamic lesions; *e. g.*, the pars anterior remained intact.

These experiments demonstrate beyond possible doubt that a moderate d.i. (35,000 cc per day for a 10 kilo dog) is possible in the absence of the pars anterior. They further, coupled with experiments already reported,[4b], are interpreted as verifying the conclusions of Trendelenburg[5] and Sato,[6] namely, that the absence of a d.i. following any hypophysectomy procedure is evidence that functional antidiuretic secretory tissue remains intact. Whether or not the elaboration of the antidiuretic principle is confined solely to hypophysial tissue is not clear.

Although a moderate d.i. is possible in the absence of the pars anterior, there is much data[7] which suggests that von Hann's concept holds to the extent that the functional presence of the pars anterior normally intensifies the severity of the d.i. resulting from a given deficit in the antidiuretic principle. However, White and Heinbecker[4c] report that in certain instances hypophysectomy did not reduce the magnitude of the d.i. induced by hypothalamic lesions. This may indicate that the above relationship does not hold when the animal is entirely antidiuretic-free.

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[5] Trendelenburg, P., *Klin. Wschr.*, 1928, **7**, 1679.

[6] Sato, G., *Arch. Exp. Path. Pharm.*, 1928, **131**, 45.

## 10272 P

## Are the Groups of Beta Hemolytic Streptococci Inter-Transformable?

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It has been reported[1] that fibrinolysis by Group A hemolytic streptococci was a character of diminishing intensity as one proceeded from the S-culture phase through the R and finally to non-hemolytic colonies showing an exclusively diphtheroid cell-morphology. Moreover, these feebly fibrinolytic diphtheroids possessed an unusual lability, as indicated by their occasional reversion to the identical S-colony type of hemolytic streptococcus (Stoddard strain) from which they had originally dissociated. Such a "reverted" diphtheroid culture agglutinated significantly in the antistreptococcal S-serum and absorbed from this serum the antibodies specific for it.

Subsequently the cultures of this diphtheroid, started from a single cell, have undergone further changes that appear to determine the Lancefield group into which the streptococcus redissociated from this diphtheroid shall fall. These changes have occurred under the following conditions. When this strain was kept for several months in our desiccated stocks and then transferred to semi-solid blood agar at room temperature, 4 transfers being made at monthly intervals, it lost practically all of its fibrinolytic ability. The same strain kept under the same conditions, except that it had been stocked on blood-agar slants in the cold room instead of being desiccated, retained its original fibrinolytic capacity. Inasmuch as Group C strains are usually non-fibrinolytic, the loss of this character might be viewed as the precursor of additional changes that resulted eventually in a strain having the serological reactions of Group C.

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[7] (a) Barnes, B. O., Regan, J. F., and Bueno, F. G., *Am. J. Physiol.*, 1933, **105**, 559; (b) Biasotti, A., *Compt. rend. Soc. de biol.*, 1934, **115**, 329; (c) Mahoney, W., and Sheeban, D., *Am. J. Physiol.*, 1933, **112**, 250; and (d) Keller, A. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 787; 1938, **38**, 31; and *Am. J. Physiol.*, 1938, **123**, 111.

[1] Mellon, R. R., and Cooper, F. B., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 451.

The non-fibrinolytic diphtheroid was then transferred to ordinary blood-agar slants which were sealed and put in the cold room (10°C). Transfers were made to the same medium under the same conditions at monthly intervals. In a future paper it will be shown that under these conditions after 5 transfers this strain underwent changes that resulted in its reversion to a hemolytic streptococcus (NF), the characters of which were obviously different from those of the streptococcus (F) that reverted under identical conditions from the fibrinolytic diphtheroid. That is to say, the F streptococcus had a 4-plus hemolysis of *beta* type in contrast with a 1-plus of *alpha* prime type for the NF strain. Moreover, the colonies were mucoid and smooth-mucoid and possessed high virulence for mice in contrast to a slight or negligible virulence of the original Group A strain (Stoddard). Fermentatively the strain had become trehalose-negative and sorbitol-positive. Thus according to this criterion the strain had assumed the status of an intermediate between Groups A and C. Antigenically it produced in animals precipitating antibodies for Group A, and was precipitated by known Group A sera. No cross-reactions were observed with Group C sera at this time.

Some 6 months later the progressive trend of this intermediate strain toward Group C became virtually complete in the sense that it acquired some antigenic characters of this group at the same time that it lost those of Group A. This change came about spontaneously while the strain was being kept on sealed blood-agar slants in the cold room. One reversion from the highly virulent intermediate C to the non-virulent Group A has been observed. This occurred *in vivo* in 2 mice that had been injected intraäbdominally with culture dilutions of $10^{-6}$ cc, which were lethal for them. In the peritoneal exudate of both the mice 2 types of colonies were obtained in about equal numbers: (a) the mucoid colony of the inoculated strain; and (b) the small SR type of colony which proved to be indistinguishable culturally and serologically from the original, slightly virulent, Group A strain.

Employing another Group A strain it has been possible to anticipate the possibility for transformations between Group A and Group C strains. This consists of broadening the range of group-antigens in a type-specific Group A strain so that its antigen agglutinates not only in several Group A serums, but also to a lesser degree in sera of Group C. This change has occurred as the result of transforming cultures of the S-phase of the type-specific culture to its corresponding R-phase and subsequently reconverting the latter to an S phase. It would thus appear that the R-phase culture, like the diphtheroid, may be the seat for reorganizations of antigenic constitution.

Both may be viewed as having something in common in respect of their proclivity for reorganization of biologic characters. The two mechanisms appear to differ in degree however, since passing through the diphtheroid state may lead to a frank recombination of characters that finds expression in a group-transformation. Further evidence for similarity between the diphtheroid and the R-culture-phase will be given fuller consideration in a subsequent publication.

The reorganizational significance of special cell-types and culture-phases was first pointed out in 1920,[2] and the special bearing of such variability phenomena on the origin of group and specific agglutinogens was indicated in 1926.[3] In 1931,[4] the same variation principles found application to the much discussed filtrable forms of bacteria—the G types, whose filtrability and marked variability have recently been confirmed by Haddow.[5]

## 10273

## A Pressor Substance is Not Present in the Perfusate of Ischemic Kidneys.

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The physiological mechanism by which increased arterial pressure is produced and maintained following the establishment of renal ischemia is unknown. There is, however, much evidence indicating that the kidney plays a specific rôle. There are several studies which seem to eliminate the possibility that reflex constriction of the peripheral vascular bed is responsible for the increased pressure observed. Since the rise in pressure may occur in the presence of one normal kidney, it seems reasonable to assume that some pressor agent may be released from the ischemic kidney in the course of an experimental hypertension. We have attempted to demonstrate such an agent in the perfusate of ischemic kidneys in hypertensive dogs.[1-5]

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[2] Mellon, R. R., *J. Med. Res.*, 1920, **42**, 61.

[3] Mellon, R. R., and Jost, E. L., *J. Immun.*, 1926, **11**, 139.

[4] Hadley, P. B., Delves, E., and Klimek, J., *J. Inf. Dis.*, 1931, **48**, 1.

[5] Haddow, A., *J. Inf. Dis.*, 1938, **63**, 129.

Two groups of dogs were used. In the first group perfusion was carried out within a few days after unilateral partial constriction of the renal artery, because we thought that any active pressor agent might be released in greatest quantity in the early stages of experimental hypertension, thereby producing increased arteriolar tonus, with subsequent maintenance of increased pressure as a result of "organic" changes in the arterioles. In the second group frank hypertension was allowed to develop.

In the first series of experiments elevation of blood pressure was produced by partially clamping the renal artery on one side and explanting the kidney into the flank. Four days later the explanted kidney was removed and perfused for a variable length of time with warm Locke's solution. The solution was perfused through the kidney several times during a period of 30 minutes in order that the total quantity of perfusate might be kept within utilizable limits. The coagulum which sometimes formed in the perfusate was removed to permit reperfusion of the fluid.

The renal perfusate was injected into the femoral vein of anesthetized dogs in which the carotid blood pressure level was recorded.

Systolic and diastolic pressures were determined in the unanesthetized state by means of the Hamilton recording optical manometer.

*Results. First Group:* In the first group of 4 animals the blood pressure ranged from 160 to 185 mm Hg when the kidney was perfused. A pressor agent was not found in the perfusate.

*Second Group:* Hypertension was produced after a control period of several weeks during which a definite blood pressure level was established. In 3 of these animals elevation of the pressure level had persisted for more than 3 months after unilateral constriction of the artery. In a fourth animal it was necessary to partially clamp the second artery in order to maintain a high blood pressure. In 2 other experiments[5] perfusion was carried out 11 and 16 days respectively after unilateral constriction of the renal artery.

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[1] Goldblatt, Lynch, Hanzal, and Summerville, *J. Exp. Med.*, 1934, **59**, 347.

[2] Blalock and Levy, *Ann. Surg.*, 1937, **106**, 826.

[3] Child and Glenn, PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 217.

[4] Alpert, Alving, and Grimson, PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 1.

[5] Williams and Grossman, *Am. J. Physiol.*, 1938, **123**, 364.

TABLE I.

| Dog No. | Pre-operative blood pressure | Pre-perfusion blood pressure | Duration of hypertension | Amt of perfusate | Duration of perfusion | Amt of perfusate injected | Anesthesia assay animal | Change in blood pressure |
|---|---|---|---|---|---|---|---|---|
| | mm Hg. | | | cc | min. | cc | | |
| 1 | 140/105 | 195/110 | 5 mo. | 150 | 30 | 70 | Pentobarbital | Transient fall |
| 2 | 150/90 | 210/112 | 4½ ” | 150 | 20 | 70 | ” | ” ” |
| 3 | 120/70 | 200/104 | 1st operation, 2 mo. 2d operation, 14 days | 200 | 20 | 100 | ” | ” ” |
| 4 | 165/105 | 250/156 | 3 mo. | 250 | 60 | 225 | Ether | Slight transient rise—8 mm Hg. |
| 5 | 142/85 | 192/112 | 11 days | 250 | 60 | 225 | ” | Slight transient rise—6 mm Hg. |
| 6 | 165/105 | 205/150 | 16 ” | 250 | 55 | 230 | ” | Transient rise—12 mm Hg. |

The results of the second group of experiments are shown in Table I. Although a definite hypertension was present, a pressor agent was not present in the perfusate. (In order to obtain the 6 hypertensive dogs, the vessels of 10 dogs were clamped.) The slight transient rises observed in dogs 4, 5, and 6 were produced when the same quantity of Locke's solution was injected at the same rate.

*Summary.* We were unable to obtain a pressor substance in significant amounts from the ischemic kidney of hypertensive dogs by perfusing such kidneys for from 20 to 60 minutes with Locke's solution.

### 10274

## Comparison of Intravaginal and Subcutaneous Tests for Estrone and Estradiol Monobenzoate.

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It was known from previous experiments[1, 2] that by administering estradiol monobenzoate intravaginally, cornification of ovariectomized rats' vaginæ could be induced with about 1/200th of the amount needed subcutaneously. It was also known that by the subcutaneous rat test, estradiol monobenzoate in oil is 5 to 10 times as potent as estrone in oil. It seemed of interest, therefore, to determine the relative potencies of these two substances as judged by the intravaginal method. For, it might be argued, that if estradiol monobenzoate appears more potent merely because of slower absorption and excretion following subcutaneous injection, the local test might find it no more potent than estrone. If, however, it is more potent because a greater growth-stimulating property is determined by its chemical structure the ratio (estradiol monobenzoate: estrone) might be expected to remain the same, or be even greater because fewer variables are involved in the local test.

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[1] Lyons, W. R., and Templeton, H. J., PROC. SOC. EXP. BIOL. AND MED., 1936, **33**, 587.

[2] Yerby, L. D., *ibid.*, 1937, **36**, 496.

Sixty adult rats of the Long-Evans strain were ovariectomized, and 2 or more weeks later were "primed" with $1\gamma$ of estradiol monobenzoate. One week later, after the vaginal reaction had subsided the higher levels of estrone and estradiol monobenzoate were tested, and then the successively lower levels, at weekly intervals. The International Standards* were dissolved in and diluted with sesame oil, and a single subcutaneous injection of 0.1 cc made. Vaginal smears were examined 48 and 72 hours later. For intravaginal tests, 0.01 cc of the various dilutions was administered on 2 successive days by means of a micropipette,[1, 2] and vaginal smears examined 24 and 48 hours after the second administration.

In both intravaginal and subcutaneous tests, a rat was considered a positive reactor if cornification was induced even though epithelial cells and some leucocytes were still present (border-line reaction). Groups of 20 rats, chosen at random, were administered a given dose and at some dose levels duplicate groups were tested by us independently. It was noticed that even on the higher doses, one or 2 rats would not show cornification, and this was usually attributable to the presence of pus.

TABLE I.
Showing the Number of Ovariectomized Rats Reacting with Vaginal Cornification to Different Levels of International Standards of Estrone and Estradiol Monobenzoate Administered Intravaginally and Subcutaneously.

| Estrone | | | Estradiol Monobenzoate | | |
|---|---|---|---|---|---|
| No. of rats | Dose in $\gamma$ | Reactors | No. of rats | Dose in $\gamma$ | Reactors |
| Subcutaneous | | | | | |
| 20 | 4.0 | 19 | 20 | .2 | 16 |
| 20 | 2.0 | 18 | 20 | .1 | 14 |
| 20 | 1.0 | 14 | 20 | .1 | 12 |
| 20 | .5 | 9 | 20 | .05 | 6 |
| 20 | .5 | 9 | | | |
| 20 | .25 | 4 | | | |
| Intravaginal | | | | | |
| 20 | .02 | 14 | 20 | .0025 | 18 |
| 20 | .02 | 12 | 20 | .0012 | 14 |
| 20 | .01 | 10 | 20 | .0006 | 12 |
| 20 | .01 | 9 | 20 | .0005 | 9 |
| 20 | .005 | 6 | 20 | .0003 | 5 |

Table I contains the results obtained from 400 tests. It will be observed that the subcutaneous rat unit (level at which at least 50% of the rats show cornification) of estrone is $0.5\text{-}1.0\gamma$ and of estradiol benzoate $0.05\text{-}0.1\gamma$. The intravaginal unit of estrone is $0.01\text{-}0.02\gamma$, and of estradiol benzoate, $0.0005\text{-}0.0006\gamma$.

Thus it may be concluded that the intravaginal unit of estrone is approximately 1/50th of the subcutaneous unit, while the intravaginal unit of estradiol monobenzoate is 1/100-1/200th of the subcutaneous unit. And whereas estradiol monobenzoate is about 10 times as potent as estrone when tested subcutaneously in rats, it is approximately 30 times as potent by the intravaginal method.

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* Kindly supplied by Dr. Oliver Kamm of Parke, Davis and Company.

### 10275

## Experimental Uterine Bleeding in Monkeys Following a Single Injection of Various Estrogens.*†

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Uterine bleeding in the rhesus monkey occurs after withdrawal of an adequate estrogenic stimulus. In routine treatments of 100 to 400 I.U. per day for 10 days, bleeding occurs in from 5 to 16 days after the last injection. In our experience, the most frequent day for bleeding of estrone withdrawal is day 7, or 17 days after the first injection.

The present series of experiments was performed with adult female macacus monkeys all of which were previously castrated. A single massive injection of an estrogen was given and the day on which uterine bleeding occurred was noted. Vaginal lavages were made daily in each animal from the time of the maximal sex skin response.

*Treatment with estradiol benzoate oleosum (Progynon B Schering).* A single injection of 150,000 I.U. (15 mg) of estradiol benzoate in oil was made. The site of injection in 2 monkeys was into the uterine cervix. By means of a vaginal speculum and adequate lighting 1 cc of the preparation was injected into the dense, fibromuscular tissue of the vaginal portion of the cervix with a number 25 hypodermic needle. The site was cleansed before and after injection with alcohol. Due to the great density of the tissue it was necessary to make 5 or 6 separate injections into the cervix around the

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† We are greatly indebted to Dr. Erwin Schwenk of the Schering Corporation for the generous supply of estradiolbenzoate; to Dr. J. A. Morrell of E. R. Squibb and Sons for the triphenylethylene, and to R. C. Mautner of the Ciba Company for the estradioldipropionate used in this study.

external os, the total dose of 1 cc thus being deposited in several pockets forced into the tissue by the pressure applied. If considerable resistance to injection does not occur, the needle is withdrawn, as a gland or the cervical canal has been entered. The purpose was to place the oily solution only in the fibromuscular coat of the cervix.

The greatest estrogenic response of the sex skin, color and edema, occurred between days 9 and 15 after the injection, after which it underwent a progressive involution. Bleeding occurred in one animal from 25 to 36 days (in 8 instances) after treatment and in the other from 21 to 49 days (Table I). The longer intervals between injection and bleeding occurred when the solution was most favorably placed in the fibromuscular stroma of the cervix.

Treatment of these animals is being continued in order to observe the effect of this type of stimulation and trauma on the uterine cervix.

The synovial bursa of the knee was used as the site of injection of 1 cc, 150,000 I.U., in 3 instances. Injection into the bursa is not difficult, although it is not possible to be certain that the entire amount is left in the bursa. The response of the sex skin was similar to that found in the intracervical injections. Bleeding occurred on days 22, 27, and 31 after the single injection.

Intraperitoneal injections caused a very slight response of the sex skin in 3 instances. Bleeding occurred on days 15 and 20, but in the third no bleeding occurred.

A single injection of estradiol benzoate will permit the typical uterine bleeding of estrone withdrawal, although the interval between the single injection and bleeding is considerably greater than that between the bleeding and the last injection in a frequently injected animal.

With this standard single injection it is clear that the rate of absorption of the hormone is a factor in regulating the length of the interval between the injection and bleeding. The site of injection giving the least satisfactory response was the intraperitoneal route, while the intrasynovial and intracervical gave the greatest interval.

*Treatment with estrabioldipropionate Ciba.* This substance is reported to cause greatly prolonged estrous smears in the female rat.[1] The response of the sex skin of the monkey is similar to that induced by other estrogens. A single injection of 1 mg of estradioldipropionate is adequate to cause the usual sex skin response and the appearance of bleeding on day 17. With 5 mg the bleeding occurred in 2 instances on days 21 and 26, while with single injec-

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[1] Unpublished data from Ciba Corporation, by courtesy of R. C. Mautner.

TABLE I.

| Monkey No. | Weight, g | Substance | Site of injection | Date of injection | Uterine bleeding days after injection |
|---|---|---|---|---|---|
| 428 | 5700 | Progynon Benzoate | Intracervical | 11/ 8/37 | 32 |
| | | 150,000 I.U. | ,, | 12/14/37 | 36 |
| | | (15 mg, 1 cc) | ,, | 1/24/38 | 27 |
| | | | ,, | 3/ 1/38 | 27 |
| | | | ,, | 4/ 4/38 | 28 (trace only) |
| | | | ,, | 5/23/38 | 25 |
| | | | ,, | 7/ 8/38 | No bleeding in 60 days |
| | | | | | Slight sex skin response |
| | | | ,, | 9/27/38 | 31 |
| 429 | 6000 | Progynon Benzoate | ,, | 10/ 7/37 | 15 |
| | | 75,000 I.U. | ,, | 11/ 8/37 | 25 |
| | | 150,000 I.U. | ,, | 12/14/37 | 32 |
| | | | ,, | 1/24/38 | 32 |
| | | | ,, | 3/ 1/38 | 21 |
| | | | ,, | 4/ 4/38 | No bleeding in 49 days |
| | | | ,, | 5/23/38 | 23 |
| | | | ,, | 7/ 8/38 | 49 |
| | | | ,, | 9/27/38 | 36 |
| 450 | 3700 | Progynon Benzoate, 150,000 I.U. | Intrasynovial | 12/22/37 | 27 |
| 452 | 3750 | ,, ,, ,, ,, | ,, | 1/24/38 | 31 |
| 480 | 6000 | ,, ,, ,, ,, | ,, | 4/ 4/38 | 22 |
| 450 | 3700 | ,, ,, ,, ,, | Intraperitoneal | 1/24/38 | 20 |
| 462 | 4350 | ,, ,, ,, ,, | ,, | 4/ 4/38 | 15 |
| 452 | 3750 | ,, ,, ,, ,, | ,, | 12/22/37 | No bleeding in 34 days |
| 463 | 5250 | ,, ,, ,, ,, | Subcutan., no massage | 1/24/38 | 16 |
| 450 | 3700 | ,, ,, ,, ,, | Subcutan., ,, ,, | 4/ 4/38 | 25 |

| | | | | | |
|---|---|---|---|---|---|
| 462 | 4350 | Estradioldipropionate Ciba (3190A), 1 mg (1 cc) | Intramuscular | 3/ 6/38 | 17 |
| 450 | 3700 | Estradioldipropionate Ciba (3190A), 5 mg (5 cc) | ,, | 3/ 2/38 | 26 |
| 481 | 5200 | Estradioldipropionate Ciba (3190A), 5 mg (5 cc) | ,, | 6/ 5/38 | 21 |
| 480 | 6000 | Estradioldipropionate Ciba (3190A), 10 mg (10 cc) | ,, | 3/ 6/38 | 30 |
| 481 | 5200 | Estradioldipropionate Ciba (3190A), 10 mg (10 cc) | ,, | 4/ 2/38 | 30 |
| 481 | 5200 | Triphenylethylene ¼ g (1.56 cc-oil) | ,, | 9/28/38 | 22 |
| 462 | 4350 | Triphenylethylene ½ g (3.12 cc-oil) | ,, | 9/28/38 | No bleeding in 55 days |
| 505 | 4450 | Triphenylethylene 1 g (6.25 cc-oil) | ,, | 9/28/38 | 33 |
| 480 | 6000 | Triphenylethylene 2 g (12.5 cc-oil) | ,, | 7/11/38 | 51 |
| 504 | 4750 | Triphenylethylene 2 g (12.5 cc-oil) | ,, | 9/ 3/38 | 67 |
| 504 | 4750 | Triphenylethylene 4 g (25 cc-oil) | ,,<br>2 g (12.5 cc-oil) at each injection | 6/16/38<br>6/20/38 | 67 |

tions of 10 mg the bleeding occurred in 2 animals on day 30, being similar to the interval obtained with 15 mg of the benzoic acid ester of estradiol (Schering), although the amount of oil was 10 times as great in the dipropionate preparation.‡

*Treatment with Triphenylethylene.* Dodds and his associates have been responsible for the development of numerous synthetic estrogenic substances, the most active of which are derivatives of stilbene.[2, 3, 4] Of these substances, triphenylethylene was used by Robson,[5] who secured typical estrogenic sex skin response and proliferative endometrium in the monkey. These reports led to the use of triphenylethylene to determine its effect on the interval between a single injection and bleeding time. A total dosage of 3.4 g was used by Robson on one monkey to produce a proliferative endometrium. One gram of triphenylethylene permits bleeding on the 33rd day, the time interval being comparable to that of 15 mg of the Progynon B. (Table I.) Two grams at a single injection delayed bleeding for 51 and 67 days respectively. Four grams also resulted in bleeding on day 67. In these instances a large amount of oil was necessary as a conveying medium. There was no local reaction to the medium. It is worthy of note that in delayed bleedings, as at 51 or 67 days, the sex skin response was maintained at a high level for about 30 days.

In this group, as with the group receiving Progynon B, recession of the sex skin took place in one instance without subsequent bleeding, a phenomenon previously related to a very slow diminution of the available estrogen.

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‡ Subsequently used with 5 mg per 1 cc.

[2] Dodds, E. C., and Lawson, W., *Nature*, 1937, **139**, 627.

[3] Dodds, E. C., Fitzgerald, M. E. H., and Lawson, W., *Nature*, 1937, **140**, 772.

[4] Dodds, E. C., Goldberg, L., Lawson, W., and Robinson, R., *Nature*, 1938, **141**, 247.

[5] Robson, J. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 153.

### 10276

## Inhibition of Tyrosinase Melanin Formation by Sodium Benzenone-Indophenol.

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It was discovered that the indophenol dyes produced a pallor in amphibian larvae raised in solutions of these dyes.[1] This suggested the possibility of solving many of the fundamental problems of pigment formation through a study of the mechanism of action of these dyes. The original work was repeated and confirmed, but extreme variation was found with regard to the effectiveness of the dyes in different species.[2] It was also proved conclusively that the dyes do not exert an influence on pigment via the hypophysis.

The present paper gives the results of experiments devised to test the hypothesis that the dyes acted directly on the pigment granules by destroying them after they were formed or by inhibiting their formation.

In preliminary experiments it was found that phenol indophenol (sodium benzenone indophenol) does not destroy pigment from the following sources: 1. Artificial pigment produced by the oxidation of either dihydroxyphenyl alanine or tyrosine. 2. Natural pigment in colloidal solution produced by brushing pigment out of the choroid coat of frozen ox eyes. 3. Natural pigment contained in melanophores in pieces of dead amphibian skin.

It was concluded from these experiments that the dye does not destroy or dissolve pigment granules after they are formed. It was thought that the dye might be inducing the pallor by influencing the enzymes responsible for pigment formation. Since tyrosinase has not been conclusively demonstrated in vertebrate forms, while dopa oxidase has, it has been assumed by many[3, 4, 5] that dopa oxidase is the enzyme responsible for pigment formation. Contrary to expectations, it was found that the dye did not inhibit the dopa reaction, but accelerated and intensified it. In addition, it accelerated the auto oxidation of dihydroxyphenyl alanine to dopa melanin.

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[1] Lewis, M. R., *J. Exp. Zool.*, 1932, **64**, 57.

[2] Figge, F. H. J., *Ibid.*, 1938, **78**, 471.

[3] Bloch, Br., and Schaaf, F., *Biochem. Z.*, 1925, **162**, 181.

[4] Laidlaw, G. F., *Anat. Rec.*, 1932, **53**, 399.

[5] Miescher, G., *Arch. f. Mikro. Anat.*, 1923, **97**, 326.

The effect of the dye was then tested on tyrosinase. It was found that the production of melanin by the action of tyrosinase on tyrosine could be either completely or partially inhibited by the dye.

Tyrosinase was obtained from mealworms *(Tenebrio mollitor)* or potatoes. The mealworm enzyme preparation was made by grinding the larvae to a fine pulp in a mortar. Ten cc of 0.25% sodium carbonate per gram of mealworm was added. This was then centrifuged in a hand centrifuge to separate the coarse particles. The gray supernatant fluid and the fine black and gray precipitates were removed from the centrifuge tubes, shaken vigorously with a few drops of chloroform, and used in this form as the active tyrosinase preparation. Part of this was then boiled to inactivate it for use in the control bottles. The potato tyrosinase was plain potato juice prepared by grating potatoes and filtering the juice through a medium coarse filter paper. The attempts to prepare concentrated enzyme preparations according to the methods of Raper,[6, 7] Gortner,[8] or Przibram[9] were not very satisfactory because the enzymes deteriorated so rapidly. The enzyme was prepared just before each experiment. The substrate was a 0.25% solution of tyrosine buffered with Sörenson's phosphate buffer mixture to pH 7.5.

It was found in the early experiments[2] on amphibian larvae that old dyes were not as effective as fresh dyes. The dye used in these experiments was manufactured a week before the initial experiment of this series. A new dye solution was made up for each individual experiment.

Fifty cc wide-mouthed bottles were used for reaction vessels. The rate of melanin formation was determined by calculating the percentage change in light absorption in any given reaction mixture. Light absorption was determined by means of a very simple photoelectric colorimeter, which made use of the same principles and resembled slightly, the Evelyn[10] photoelectric colorimeter.

The reaction bottles are listed in the table in pairs according to the concentration of the enzyme. One of the bottles of each pair contains, in addition to the substrate and enzyme, the quantity of dye indicated in the table. The degree of inhibition for any dye/enzyme ratio may be estimated by comparing the percentage change in light absorption in any given pair of reaction bottles. At the end of the first hour the bottles containing enzyme and substrate only showed

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[6] Raper, H. S., *Biochem. J.*, 1926, **20**, 725.

[7] Raper, H. S., and Wormall, A., *Ibid.*, 1923, **17**, 454.

[8] Gortner, R. A., *J. Biol. Chem.*, 1911-12, **10**, 89.

[9] Przibram, H., and Brecher, L., *Arch. f. Entw-mech.*, 1919, **45**, 83.

[10] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

a change in percentage light absorption ranging from 9.5% to 45%. None of the "d" bottles containing dye showed any change. It is, therefore, assumed that all concentrations of dye completely inhibited pigment formation during this period. The figures on percentage change in light absorption after longer time intervals showed that the enzyme tyrosinase was completely inhibited in the bottles containing small amounts of enzyme and large amounts of dye. The smaller quantities of dye with larger amounts of enzyme only partially inhibited the enzyme.

TABLE I.

| Bottle No. | 15 cc substrate | | Rate of melanin formation as indicated by percentage light absorption at end of | | |
|---|---|---|---|---|---|
| | cc enzyme | mg dye | 1 hr | 5 hr | 14 hr |
| 1 | .1 | | 9.5 | 68 | 92 |
| 1 d | .1 | .5 | 0 | 0 | 0 |
| 2 | .2 | | 10 | 78 | 94 |
| 2 d | .2 | .4 | 0 | 0 | 0 |
| 3 | .3 | | 20 | 84 | 97 |
| 3 d | .3 | .3 | 0 | 0 | 36 |
| 4 | .4 | | 39 | 91 | 98 |
| 4 d | .4 | .2 | 0 | 0 | 50 |
| 5 | .5 | | 45 | 94 | 98 |
| 5 d | .5 | .1 | 0 | 32 | 77 |

This experiment was repeated 5 times, using mealworm tyrosinase and once with potato tyrosinase. Readings were made at one or 2 hour intervals. The plotted curves for all experiments were essentially uniform and the results were in such close agreement with the quantitative variations in the 5 different concentration ratios that further repetition was deemed unnecessary.

The conclusion to be drawn from this is that sodium benzenone indophenol inhibits the enzyme tyrosinase and thus prevents the formation of pigment granules. The pallor which this and similar dyes induce in young amphibian larvae may be explained on this basis. This indicates that even though it has been impossible as yet to demonstrate tyrosinase in these forms, this enzyme, and not dopa oxidase, must be responsible for most of the melanin formation in young larval amphibians.

### 10277 P

## A Highly Fatal Disease of Guinea Pigs.

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Our guinea pig colony is carefully watched for infectious diseases and every attempt is made to eliminate any that appear. For several years deaths have been few and the common guinea-pig pathogens have not been observed. This year, in a group of apparently normal guinea pigs removed for observation, a few young animals were found to have elevated temperatures. Inoculations of organ-suspensions were made into other animals and an agent was secured that is highly fatal for guinea pigs.

When injected subcutaneously, intraperitoneally, intracerebrally, or intranasally, guinea pigs show an elevated temperature in from 2 to 5 days, which persists for approximately 5 days and then drops to normal or below. Leucocyte counts show from 3000 to 5000 cells, while the red corpuscles are normal in number and volume. The infection can be transmitted by contact, but so far attempts to infect by feeding have been unsuccessful. Guinea pigs die in about 2 weeks after inoculation, and to date not a single animal has survived.

Autopsy shows a loss of subcutaneous fat, with dilated blood vessels in the subcutis. Animals weighing 250 g will lose from 50 to 75 g, while those weighing 500 g will lose from 125 to 175 g. The kneefold lymph-nodes are enlarged and reddened. The stomach is collapsed and the abdominal cavity appears too large for its contents. The abdominal and thoracic viscera show no characteristic gross pathology. Pneumonia is absent except in animals inoculated intranasally and those infected by contact.

When guinea pigs carrying hemolytic streptococcus or *B. bronchisepticus* are inoculated extensive pneumonia is usually found at autopsy. The combined infection of hemolytic streptococcus and the agent can be transmitted by contact and in exposed animals the pulmonary lesions are the predominating feature. Exposure-experiments with *B. bronchisepticus* and the agent have not been done.

Young white mice are not favorable animals for use, since after intracerebral, intranasal, or intraperitoneal injection only a portion die. Intracerebral injection into rabbits produces a fever of short duration. Cutaneous inoculation of rabbits causes no local lesion. Intratesticular injection has resulted in an orchitis in one animal;

subinoculations from this animal have produced orchitis in all injected rabbits, and the testicular suspension injected into guinea pigs has caused the disease described above. Intraperitoneal injection into 2 cats has been negative.

The agent causing this infection is in suspensions of various organs, in tracheal washings, and in the blood serum. Titrations of sera from infected guinea pigs have shown that from $1 \times 10^{-3}$ to $1 \times 10^{-5}$ cc will cause disease. Heating of infective serum for 20 minutes at 50°C has not destroyed its activity. Serum heated at 55°C for the same time gave a prolonged incubation period, while serum heated for 20 minutes at 60°C was inactive.

Cultures of infective organ suspensions or serum show no growth on ordinary media. The agent readily passes through tested Berkefeld N filters, and Berkefeld W filtrates cause disease but death occurs 2 to 3 days later than in the controls. The agent has been propagated on the chorioallantoic membrane of embryonated chicken eggs, and suspensions of membranes from the 18th serial transfer have produced the characteristic disease in guinea pigs.

The source of this infection has not been determined. It is probably not of human origin, for the sera of the 2 caretakers and of 4 of us who have been working with the disease all fail to neutralize the agent.

This agent appears to be a filtrable virus capable of producing disease by itself as well as in association with the common guinea-pig pathogens.

## 10278

## Metabolism of the Alcoholate of the Trimer of Hydroxypyruvic Aldehyde.

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Hydroxypyruvic aldehyde has been prepared previously by Evans and Waring[1] and by Hynd.[2] Hynd also studied its toxicity and action in insulin shock. Elsewhere the authors[3] have described a new method for the purification and isolation of the alcoholate of its trimer $[(C_3H_4O_3)_3.C_2H_5OH]$.

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[1] Evans, W. L., and Waring, C. E., *J. A. C. S.*, 1926, **48**, 2678.

[2] Hynd, A., *Biochem. J.*, 1931, **25**, 11.

[3] Evans, W. E., Jr., Carr, C. J., and Krantz, J. C., Jr., *J. A. C. S.*, 1938, **60**, 1628.

*Glycogen Storage in the Livers of White Rats.* Nine male animals fasted for 48 hours were given 0.4 g of hydroxypyruvic aldehyde per 100 g of animal. The compound was dissolved in 3 cc of water and administered by stomach tube. After 3 hours the rats were anesthetized with sodium amytal, the livers extirpated and the glycogen determined by the Good[4] modification of Pflüger's method. The dextrose was determined by the Shaffer and Hartmann[5] method. The low value was 0.11%, high 0.62%, and the average 0.29% liver glycogen. Four control animals which received 3 cc of water showed an average liver-glycogen content of 0.28%.

Under the same experimental conditions 6 additional animals were given 0.4 g of dihydroxyacetone per 100 g of animal. The average liver-glycogen content was 1.01%.

In order to eliminate the possibility of slow absorption of hydroxypyruvic aldehyde from the alimentary tract and consequent lack of effect on liver-glycogen content, doses of 0.25 g per 100 g and 0.025 g per 100 g were administered intraperitoneally to 9 and 10 rats respectively. Six animals which had received 1 cc of Ringer's solution intraperitoneally served as controls. Thirty minutes after injection, the liver-glycogen content was determined. The controls averaged 0.30% glycogen. Those which had received 0.25 g of hydroxypyruvic aldehyde per 100 g averaged 0.19% glycogen, and the livers of the rats which were injected with 0.025 g of the osone per 100 g averaged 0.11%. Under these conditions hydroxypyruvic aldehyde does not serve as a precursor of glycogen.

*Blood Sugar.* All animals were fasted 24 hours prior to treatment. Doses of 4 g of dextrose per kg and 4 g of the glycerosone per kg were given by stomach tube to 7 and 10 rabbits respectively. The blood sugar was determined by the Folin[6] ferricyanide method. No differentiation was made between the blood dextrose and blood hydroxypyruvic aldehyde. The quantitative reducing power of the osone compound was 30% less than that of dextrose. The effects on blood-sugar level are summarized in Table I. After hydroxypyruvic aldehyde a mild glycemia existed for 6 or 7 hours; the value was normal the following day.

The effect on the blood sugar level of the intravenous administration of 0.25 g per kg of hydroxypyruvic aldehyde and dex-

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[4] Good, C. A., Kraemer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[5] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, **45**, 349.

[6] Folin, O., *J. Biol. Chem.*, 1928, **77**, 421.

TABLE I.

**Effect of Hydroxypyruvic Aldehyde on Blood-Sugar Level of Rabbits.**

| No. of Rabbits | Substance Administered Dose = 4 g per kg | Oral Administration Blood-Sugar Level (avg mg %) Time in Hours | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | ½ hr | 1 hr | 2 hr | 3 hr | 4 hr |
| 7 | Dextrose | 120 | 177 | 205 | 164 | 145 | 128 |
| 10 | Glycerosone | 107 | 120 | 112 | 120 | 123 | 134 |

TABLE II.

Effect of Hydroxypyruvic Aldehyde on the Blood-Sugar Level of Rabbits.

| No. of Rabbits | Substance Administered Dose = 0.25 g per kg | Intravenous Administration Blood-Sugar Level (avg mg %) Time in Min. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 60 | 90 | 120 |
| 4 | Dextrose | 107 | 205 | 190 | 199 | 188 | 168 | 172 | 168 | 168 | 153 | 158 | 128 | 116 | 116 |
| 10 | Glycerosone | 109 | 106 | 102 | 121 | 112 | 100 | 104 | 100 | 104 | 119 | 116 | 122 | 128 | 123 |

trose was determined. The results are summarized in Table II.

Intravenous injection of 0.5 to 1.0 g of hydroxypyruvic aldehyde per kg produced immediately the following symptoms: transient exophthalmus, respiratory stimulation both in rate and depth, and ischemia of the ears for varying periods of time. Clonic convulsions ensued, characterized by dorsiflexion of the head, with intervening periods of muscular weakness of the extremities especially the hind legs. Expressed urine samples did not reduce Fehling's solution. The foregoing symptoms were relieved or prevented by the subsequent or simultaneous administration respectively of 5 cc per kg of a 5% solution of disodium phosphate intravenously. Dextrose administered under the same conditions produced extreme dilatation of the ear veins. Urine expressed a short time after the injection reduced Fehling's solution strongly upon heating.

*Insulin Shock.* Thirty-five mice, weighing about 20 g each, were fasted for 24 hours. Each was then injected intraperitoneally with 1/6 unit of insulin and placed in a chamber heated to 29°. Ten of these animals served as controls. The intraperitoneal injection of 2.0 mg per gram of hydroxypyruvic aldehyde in 4% solution into each of 15 animals relieved the convulsions but this was succeeded by depression characterized by muscular weakness and labored respiration, followed by death. The insulin convulsions were relieved for the greatest period of time by the intraperitoneal injection of 1 mg per gram of the osone into each of 6 mice. These animals later died in convulsions which were typical of insulin. Hynd[7] reported that the injection of hydroxypyruvic aldehyde did not relieve insulin convulsions.

The injection of 0.25 g per kg of hydroxypyruvic aldehyde into a totally depancreatized dog caused temporary relief of convulsions produced by protamine zinc insulin.

*Conclusions.* 1. The oxidation of one primary alcoholic group in dihydroxyacetone with the production of hydroxypyruvic aldehyde destroys the capacity of the former compound to be stored as glycogen in the liver of the rat. 2. The oral administration or intravenous injection of the osone does not significantly affect the blood-sugar level of rabbits within 2 hours. 3. The intraperitoneal injection of hydroxypyruvic aldehyde into mice causes temporary recovery from insulin convulsions, although it is not a precursor of glucose.

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[7] Hynd, A., *Proc. Roy. Soc. London*, 1927, B **101**, 244.

## 10279

# Studies in Diuresis with Isomannide. Sugar Alcohols XVIII.

JOHN C. KRANTZ, JR., AND C. JELLEFF CARR.

*From the Department of Pharmacology, School of Medicine, University of Maryland.*

In previous communications the authors[1, 2, 3] showed that isomannide (1,4,3,6 anhydromannitol) when fed or administered by stomach tube to the white rat failed to give rise to additional liver glycogen. In the feeding experiments large quantities of unchanged isomannide were observed to crystallize from the evaporated urine of animals, besides the compound was strikingly innocuous. The non-toxic nature of isomannide and its physical and physiological properties suggested its use as a diuretic. The work of Bullock, *et al.*,[4] on the use of intravenous sucrose as a diuretic in the treatment of intracranial pressure and the work of West and Burget[5] on the use of sorbitol as a diuretic prompted the study of the effect of isomannide on urine flow. Isomannide (Fauconnier[6]), a white crystalline solid, melts between 86° and 87°C. The compound is dextrorotatory; $[\alpha]^{20} + 91.36$. At room temperature 900 g may be dissolved in sufficient water to make a liter. In aqueous solution the molecules are not appreciably hydrated or associated as evinced by cryoscopic determinations. The pH of a 10% solution in boiled distilled water is approximately 7.2.

*Diuresis in Dogs Intravenously.* Animals anesthetized with nembutal (32 experiments) were prepared for urine flow determinations by cannulating the ureters with a V-shaped cannula. Urine volumes were measured. Isomannide was injected in a 50% solution on the basis of 2.5 cc per kg after a uniform flow had been established. The total volume was injected within a period of 5 minutes. Graph 1 shows the average diuresis effect from the injection of isomannide intravenously in successive doses.

*Diuresis in Dogs Orally.* The foregoing experiment was repeated excepting that isomannide was injected through the stomach wall

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[1] Carr, C. J., Musser, R., Schmidt, J. E., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1933, **102**, 721.

[2] Carr, C. J., Krantz, J. C., Jr., *J. Biol. Chem.*, 1938, **124**, 221.

[3] Krantz, J. C., Jr., Evans, W. E., Jr., and Carr, C. J., *Quart. J. Pharm. and Pharmacol.*, 1935, **8**, 213.

[4] Bullock, L. T., Gregersen, M. I., and Kinney, I., *Am. J. Physiol.*, 1935, **112**, 82.

[5] West, E. S., and Burget, G. E., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 105.

[6] Fauconnier, A., *Bull. Soc. Chim. Paris*, 1884, **41**, 119.

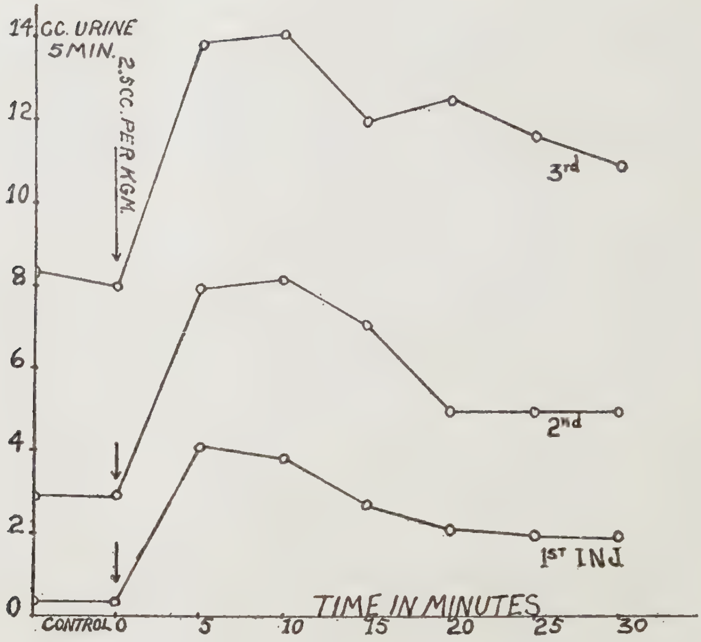

FIG. 1.
Diuresis in Dogs by Isomannide (Intravenously).

instead of intravenously. The effect of an equal volume of water administered in the same manner served as a control. Definite diuresis was produced by isomannide in 6 experiments.

*Isomannide Excretion.* The strongly dextrorotatory properties of isomannide permit accurate determinations polarimetrically in optically inactive urine. It was found that standard solutions of ceric sulfate in the presence of sulfuric acid quantitatively oxidized isomannide. The excess ceric sulfate reacts with potassium iodide and the residual oxidizing reagent can be titrated as free iodine with standard thiosulfate solutions. The organic non-protein constituents in the tungstic acid blood filtrate reduce also the ceric sulfate solution and corrections must be made for this factor on each animal. In the present state of refinement of this method the authors are able to determine 0.1 mg of isomannide in 0.5 cc of blood within an accuracy of ±2.5% of the concentration of isomannide present. The foregoing intravenous experiment on the dog was repeated (5 experiments) and the urinary and blood isomannide concentrations determined at various intervals. Graph 2 shows 2 typical experiments on the rate of clearance from the blood of isomannide by intravenous injection in the dog (2.5 cc of a 50% solution per kg).

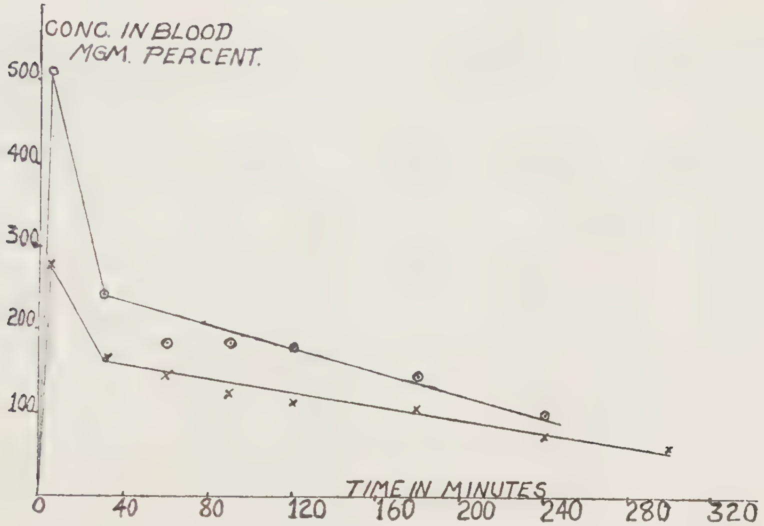

FIG. 2.
**Excretion of Isomannide from Dog's Blood. Two Typical Experiments.**

*Man.* Four normal males were given approximately the same food and water intake for 2 days. On the second day 10 g of isomannide were ingested dissolved in 50 cc of water. Urinary findings and isomannide excretion are shown in Table I. Two normal individuals were given accurately weighed diets which contained protein 75 g, fat 100 g, carbohydrate 250 g, salt 5 g, caloric value 2,500, and added water to make a total of 2 liters. Their emotional and physical behavior was similar on all 4 days. No precipitous changes in the temperature or humidity occurred during the experiment. On the third and fourth days 10 g of isomannide were administered in the morning and 3 g every 3 hours for 3 doses. Urinary findings and isomannide excretion are shown in Table I.

*Toxicity.* Dogs were given 2.5 cc per kg (15 experiments) of a 50% solution intravenously without demonstrable gross effects. An 11 kg monkey *(Rhesus macacus)* was given 20 cc of a 50% solution intravenously apparently without effect. In man (15 experiments) as much as 20 g have been administered by mouth without symptoms of gastrointestinal irritation. Two rats were fed a standard laboratory ration for 4 days and then 0.25 g of isomannide was added to the food of each animal each day. This was continued for 14 days. The animals did not lose weight or show toxic symptoms during this period. The animals were sacrificed and their kidneys and livers were examined grossly and histologically. There was no evidence of kidney or liver damage. The foregoing procedure was repeated

TABLE I.
Isomannide Diuresis in Man.

| Subject | Urine volume, cc | Sp. Gr. | pH | Total NaCl g | Total $N_2$ g | Isomannide ingested g | % excretion 24 hours |
|---|---|---|---|---|---|---|---|
| L.Y. | 750 | 1.030 | 5.50 | 10.80 | 12.40 | 0 | 0 |
| L.Y. | 952 | 1.030 | 5.16 | 14.70 | 12.52 | 10 | 68.4 |
| W.E. | 805 | 1.024 | 5.78 | 9.20 | 10.17 | 0 | 0 |
| W.E. | 1000 | 1.022 | 5.85 | 12.20 | 9.95 | 10 | 57.7 |
| H.W. | 780 | 1.023 | 5.57 | 6.80 | 11.85 | 0 | 0 |
| H.W. | 1112 | 1.021 | 5.68 | 11.00 | 12.40 | 10 | 70.7 |
| C.J. | 835 | 1.022 | 6.20 | 10.50 | 8.95 | 0 | 0 |
| C.J. | 1551 | 1.015 | 6.03 | 11.00 | 8.80 | 10 | 90.0 |
| L.Y. | 840 | 1.024 | 5.91 | 10.81 | 10.53 | 0 | 0 |
| L.Y. | 620 | 1.026 | 5.42 | 5.10 | 10.45 | 0 | 0 |
| L.Y. | 886 | 1.028 | 5.48 | 6.05 | 12.95 | 19 | 74.1 |
| L.Y. | 677 | 1.035 | 5.42 | 4.20 | 11.40 | 19 | 94.5 |
| H.W. | 890 | 1.018 | 7.21 | 5.12 | 9.70 | 0 | 0 |
| H.W. | 920 | 1.022 | 6.32 | 5.71 | 9.60 | 0 | 0 |
| H.W. | 800 | 1.025 | 5.68 | 5.24 | 11.27 | 19 | 63.4 |
| H.W. | 1425 | 1.015 | 7.45 | 5.78 | 10.96 | 19 | 119.0* |
| Control Mean | 805 | 1.024 | 5.97 | 8.01 | 10.45 | | |
| Isomannide Mean | 1050 | 1.024 | 5.84 | 8.77 | 11.28 | | 79.7 |

*Excretion from preceding day.

on 2 additional animals for 14 days and daily examinations were made of the urine for hemoglobin. All tests were negative.

*Effect Upon Blood Constituents.* Isomannide injections did not produce methemoglobin in the dog as shown by spectrophotometric analysis. Solutions of isomannide made isotonic with blood were found to rapidly hemolyze red blood cells (rabbit, dog and man). This effect, similar to urea, is not due to damage to the cell and is entirely an osmotic pressure phenomenon and not erythrolytic as shown by the fact that hemolysis is completely prevented by the presence of isotonic salt solution. In certain animals a transient hemoglobinuria is produced by massive injections of isomannide solutions. When this compound is injected dissolved in normal salt solution the production of hemoglobinuria is less frequent and appears only after repeated administration. The authors have never observed hemoglobinuria in animals, or with massive doses in man, when isomannide is given orally. The clearance from the blood of isomannide as shown in Graph 2 demonstrates the rapid diminution of the amount in the dog's blood until a concentration of the order of magnitude of 180 mg % is reached. At this point the diminution follows a die-away curve with the slow elimination of isomannide and continued diuresis. In the dog, after the intravenous injection of isomannide solution the concentration of the compound in the urine was found as high as 10%. At this time the concentration in the blood was approximately 0.2%, which indicates the capacity of the kidneys of some dogs to concentrate this compound 50 fold. The great solubility of isomannide in water permits ready absorption from the alimentary tract in man, dogs and rats, producing diuresis rather than catharsis as does mannitol.

*Summary.* Isomannide when administered orally to man or by vein to dogs is excreted unchanged in the urine in large quantities producing an increase in urine volume. Isomannide when ingested in large quantities or administered intravenously produced no toxic symptoms. Like urea, isomannide permeates the red blood cells eliciting the characteristic osmotic phenomena. The diuretic activity of isomannide is likely due to its lack of tubular absorption, thus increasing the osmotic pressure of the fluid upon which the water-absorbing cells are acting and thereby increasing the volume of urine excreted.

## 10280

## Sulfanilamide and Derivatives in the Treatment of Experimental Tuberculosis of Guinea Pigs.

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Sulfanilamide has been found by Rich and Follis[1] to exert a markedly inhibitory effect upon experimental tuberculosis of guinea pigs when given in 2 daily doses of 0.1 g and 0.5 g divided into 4 doses. Treatment was started 3 days before the subcutaneous injection of human tubercle bacilli. Smithburn,[2] however, failed to observe any beneficial effects from the daily administration of 0.2 g to guinea pigs by intraperitoneal injection 24 hours before the intracerebral inoculation of virulent human bacilli. Greey, Campbell and Culley[3] have reported that sulfanilamide (Prontylin powder) in 3 daily doses of 0.1 g each *per os* started 3 days before subcutaneous inoculation with virulent human bacilli had an inhibitory effect upon the development of lesions; when the infection had been present 17 to 24 days, treatment did not alter the macroscopic appearance of the lesions. The development of sensitivity to tuberculin was unaltered by sulfanilamide therapy.

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[1] Rich, A. R., and Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 77.

[2] Smithburn, K. C., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 574.

[3] Greey, P. H., Campbell, H. H., and Culley, A. W., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 22.

Sixteen guinea pigs were inoculated subcutaneously in the region of the inguinal lymph nodes with 0.00002 g of virulent tubercle bacilli; 4 were kept as untreated controls. Treatment of the remaining 12 was begun 2 hours after inoculation; 4 were given 0.2 g sulfanilamide per kilo divided into 2 doses 6 hours apart by intramuscular injection daily for 8 days (16 doses); 4 were given 0.3 g and 4 were given 0.5 g per kilo divided into 2 doses 6 hours apart daily for the same period. Three of the untreated controls died in 39 to 63 days after inoculation with well developed tuberculous lesions in the lymph nodes, spleen, liver and lungs, showing tubercle bacilli in smears and confirmed by microscopical examination of the tissues; the fourth was killed on the 66th day for autopsy purposes. Of the 12 treated animals 10 died in 23 to 65 days after inoculation and the remaining 2 were killed on the 65th day for autopsy purposes. All showed tuberculous adenitis and splenitis of a degree comparable to the untreated controls; 10 showed tuberculous infection of the liver and 8 of the lungs, confirmed by positive smears for tubercle bacilli and by microscopical examination of the tissues (Table I). In general terms we have interpreted the results as indicating that sulfanilamide had no curative activity under the conditions of the experiment.

Thirty-seven guinea pigs were inoculated with 0.00002 g virulent human tubercle bacilli by subcutaneous injection in the region of the inguinal lymph nodes; 7 were kept as untreated controls. Treatment was started 9 days later, at a time when the glands showed first evidences of infection, with the following 6 derivatives of sulfanilamide in the form of sodium salts:

| | | | | | | |
|---|---|---|---|---|---|---|
| No. 2219 | p-sulfonamide | benzene | azo | (1′-naphthylamine-4′-sulfonic | acid). | |
| No. 2247 | ” | ” | ” | (1-naphthol-5 | ” | ” ). |
| No. 2251 | ” | ” | ” | (1-naphthol-4 | ” | ” ). |
| No. 2253 | ” | ” | ” | (2-naphthol-6 | ” | ” ). |
| No. 2274 | ” | ” | ” | (chloro-resorcinol). | | |
| No. 2306 | Sodium sulfanilamide. | | | | | |

Each of these compounds was tolerated by mice and rabbits in an average dose of 1.5 g per kilo administered subcutaneously and *per os*.

TABLE I.
Sulfanilamide in the Treatment of Experimental Tuberculosis of Guinea Pigs.*

| Pig No. | Dose per kilo† g | Death (Days after Inoc.) | Autopsy Findings | | | |
|---|---|---|---|---|---|---|
| | | | Lymph Nodes | Spleen | Liver | Lungs |
| 1 | .2 | 39 | ‡+ | + | — | — |
| 2 | ” | 56 | + | + | — | + |
| 3 | ” | 23 | ++ | ++ | ++ | + |
| 4 | ” | 47 | + | ++ | + | + |
| 5 | .3 | 60 | ++ | ++ | + | ++ |
| 6 | ” | 61 | ++ | ++ | + | ++ |
| 7 | ” | 65§ | + | ++ | + | — |
| 8 | ” | 58 | + | ++ | + | + |
| 9 | .5 | 64 | + | ++ | ++ | + |
| 10 | ” | 65§ | + | ++ | + | — |
| 11 | ” | 65 | + | ++ | + | — |
| 12 | ” | 62 | + | ++ | + | + |
| 13 | Control | 39 | + | ++ | ++ | ++ |
| 14 | ” | 58 | + | ++ | + | — |
| 15 | ” | 63 | + | ++ | ++ | + |
| 16 | ” | 66§ | + | + | + | + |

*Animals inoculated with 0.00002 g human tubercle bacilli by subcutaneous injection in inguinal region.

†Divided into 2 doses given daily 6 hours apart for a total of 8 days (16 doses) by intramuscular injection. First dose given 2 hours after inoculation.

‡+ = slight tuberculous infection with few tubercle bacilli in smears of lesions; ++ = heavy infection.

§Killed for autopsy purposes.

The doses given per kilo of weight are shown in Table II. Each was divided into 2 parts given 6 hours apart by intramuscular injection daily for 6 days; then every other day for 4 days, followed by treatment every 3 days for 4 days, totalling 28 doses.

Five of the 7 untreated controls died in 23 to 50 days after inoculation and the remaining 2 were killed on the 43rd day for autopsy purposes. All showed severe tuberculous adenitis and splenitis, confirmed by direct smears of the lesions for tubercle bacilli and by microscopical examination of the tissues; all showed infection of the liver and 6 showed macroscopic infection of the lungs.

Twenty-five of the 30 treated animals died in 25 to 46 days after inoculation and the remaining 5 were killed on the 46th day for autopsy purposes. All showed tuberculous adenitis, confirmed by positive smears and microscopical examination of the tissues; 29 showed confirmed macroscopic lesions of the spleen, liver and lungs. Our interpretation of the results indicated that the 6 derivatives of sulfanilamide employed were without beneficial effects under the conditions of the experiment.

*Conclusions.* (1) Sulfanilamide by intramuscular administration was without demonstrable beneficial effect in the treatment of experimental tuberculosis of guinea pigs when begun 2 hours after inocu-

TABLE II.
Derivatives of Sulfanilamide in the Treatment of Experimental Tuberculosis of Guinea Pigs.*

| Compound No. | Dose per kilo† g | Death (Days after Inoc.) | Autopsy Findings | | | |
|---|---|---|---|---|---|---|
| | | | Lymph Nodes | Spleen | Liver | Lungs |
| 2219 | 1.0 | 40 | ‡++ | + | + | ++ |
| ” | ” | 37 | ++ | + | + | ++ |
| ” | 2.0 | 38 | + | — | — | — |
| ” | ” | 34 | ++ | ++ | + | + |
| ” | ” | 46§ | + | + | + | + |
| 2247 | 1.0 | 31 | ++ | ++ | + | + |
| ” | ” | 40 | ++ | + | ++ | + |
| ” | 2.0 | 38 | ++ | ++ | + | + |
| ” | ” | 37 | ++ | ++ | + | + |
| ” | ” | 36 | ++ | + | + | + |
| 2251 | 0.1 | 46§ | ++ | ++ | ++ | ++ |
| ” | ” | 41 | ++ | + | + | + |
| ” | 0.2 | 39 | ++ | ++ | ++ | + |
| ” | ” | 35 | + | ++ | + | + |
| ” | ” | 34 | ++ | ++ | ++ | ++ |
| 2253 | 0.1 | 46§ | ++ | + | ++ | + |
| ” | ” | 25 | + | ++ | + | + |
| ” | 0.2 | 46§ | + | + | + | + |
| ” | ” | 45 | ++ | + | + | + |
| ” | ” | 46 | ++ | + | + | + |
| 2274 | 0.1 | 33 | + | + | + | + |
| ” | ” | 27 | ++ | ++ | ++ | + |
| ” | 0.2 | 32 | ++ | ++ | ++ | ++ |
| ” | ” | 26 | ++ | ++ | ++ | ++ |
| ” | ” | 26 | ++ | + | + | ++ |
| 2306 | 1.0 | 46§ | ++ | + | + | + |
| ” | ” | 36 | + | + | + | + |
| ” | 2.0 | 36 | + | + | + | ++ |
| ” | ” | 40 | + | + | + | + |
| ” | ” | 39 | + | + | + | + |
| Control | — | 32 | ++ | ++ | + | + |
| ” | — | 43§ | ++ | ++ | ++ | ++ |
| ” | — | 25 | ++ | ++ | ++ | + |
| ” | — | 31 | ++ | + | ++ | + |
| ” | — | 43§ | ++ | ++ | ++ | + |
| ” | — | 50 | ++ | ++ | + | — |
| ” | — | 23 | ++ | + | + | + |

*Animals inoculated with 0.00002 g human tubercle bacilli by subcutaneous injection in inguinal region.

†Divided into 2 doses given daily 6 hours apart for 6 days; then every other day for 4 days, followed by treatment every 3 days for 4 days of treatment (total 28 doses). Treatment started 9 days after inoculation.

‡+ = slight tuberculous infection with positive smears for tubercle bacilli; ++ = heavy infection.

§Killed for autopsy purposes.

lation. (2) Six derivatives of sulfanilamide in the form of their sodium salts, by intramuscular injection, were without demonstrable curative effects in the treatment of experimental tuberculosis of guinea pigs when treatment was started 9 days after inoculation.

## 10281

# Deterioration of Staphylococcal Toxin in Saline.

R. H. RIGDON AND HENRY HARRIS. (Introduced by R. C. Avery.)

*From the Department of Pathology, Vanderbilt University Medical School, Nashville, Tenn.*

There is some evidence of union between certain bacterial toxins and susceptible tissues. The harmful effects of tetanal toxin are neutralized when it is mixed *in vitro* with brain and cord tissues of rabbits and guinea pigs as shown by Wassermann and Takaki.[1] Metchnikoff observed that brain-tissue from animals entirely insusceptible to tetanal toxin possesses absolutely no neutralizing properties.[2]

Doerr[3] found that rabbits' spinal cord macerated in saline had little effect on the lethal action of tetanal toxin.

The action of some toxins on certain tissues *in vivo* endangers the life of the animal. The injury produced in connective tissue by diphtheric toxin, ricin, and abrin can only be explained by anchoring of the toxin on the cells, according to Dean.[4]

Staphylococcal toxin when injected intravenously in the rabbit may produce immediate death.[5] If the animal survives the acute reaction, necrosis may occur in the majority of the organs.[6] When the toxin is injected locally necrosis occurs in the immediate area.[7]

The present study was made to determine the *in vitro* effect of rabbit brain, liver, kidney, and muscle on a staphylococcal toxin. The tissues were macerated and mixed immediately with the toxin and kept at 5-15°C for 24 hours. The toxin-tissue mixtures were centrifuged and the supernatant liquid was titrated for hemotoxin with 1 cc of a 2% suspension of rabbits' red blood cells. Lysis was recorded after the tubes had been in a waterbath at 37°C for 2 hours and in the icebox for 18 hours. The degrees of hemolysis in the different tubes were compared with that produced by a known amount of toxin diluted with saline.

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[1] Wassermann, A., and Takaki, T., *Berl. Klin. Woch.*, 1898, **35**, 5.

[2] Metchnikoff, E., *Ann. de l'Inst. Past.*, 1898, **12**, 81.

[3] Doerr, R., *Hyg. und Infektionshkr.*, 1936, **118**, 212.

[4] Nuttall, C. H. F., and Graham-Smith, G. S., *The Bacteriology of Diphtheria*, Cambridge University Press, 1913, p. 546.

[5] Kraus, R., and Pribram, E., *Wien. Klin. Wchnschr.*, 1906, **19**, 493.

[6] Rigdon, R. H., *Arch. Path.*, 1935, **20**, 201.

[7] Rigdon, R. H., *Arch. Path.*, 1937, **23**, 634.

Table I shows variation in the effect of staphylococcal toxin of different tissues from the same rabbit and from a different rabbit. The supernatant liquids from the tissues of rabbit A have more toxin than the control diluted with saline. The amount of hemotoxin recovered from the liver and brain of rabbit B is more than that in the saline control. However, the supernatant fluid from the muscle and kidney contains less than the saline control.

TABLE I.
**Effect Produced on Hemolysin by Mixing Staphylococcal Toxin with Rabbit Tissues Macerated in Saline.**

| Tissue | Rabbit A | | | Rabbit B | | |
|---|---|---|---|---|---|---|
| | Hemolysin in mm$^3$ necessary to produce same degree of lysis | | Avg amt of hemolysin | Hemolysin in mm$^3$ necessary to produce same degree of lysis | | Avg amt of hemolysin |
| Kidney | .3<br>.4 | .5<br>.6 | .45 | .8 | .1 | .9 |
| Liver | .2<br>.3 | .3<br>.4 | .3 | .3 | .4 | .35 |
| Brain | .23<br>.25 | .35<br>.35 | .295 | .3 | .4 | .35 |
| Muscle | .32 | .55 | .435 | .6 | 1.1 | .85 |
| Saline Control | .5 | .8 | .65 | | | |

The experiment was repeated. Infusion-broth, pH 7.4, was substituted for the saline. The results were quite similar.

The 2 experiments show that more hemotoxin is present in the supernatant fluid from the livers and brains of the 4 rabbits than in the control mixtures of toxin with broth and with saline. There is less hemotoxin from the muscle in 3 of the 4 animals and less from the kidney in 2 of the 4 rabbits. These results suggest that the toxin may deteriorate either when diluted with saline or in the presence of macerated muscle and kidney tissues. Other tissues appear to enhance the hemolytic activity of the toxin toward rabbits' red blood cells.

The effect of extracts of rabbit tissues on staphylococcal toxin was studied to obtain additional information. The same types of tissues as previously used were extracted in saline and also in infusion-broth. The toxin was added and the preparations were kept at 5-15°C for 18 hours. The toxin-extract mixtures were then titrated for hemotoxin. The results show that approximately the same amount of hemotoxin is recovered from the extracts of kidney, liver, and brain as from the controls diluted with an equal quantity of broth. There is, however, in the extracts of kidney, liver, and brain more hemotoxin than in the controls diluted with saline. The amount of toxin recovered from the extract of muscle is less than that recovered from the other tissue-extracts and also less than that in both of the controls. The results of all the experiments suggest that the variation in the amount of hemotoxin recovered from either macerated tissue or tissue-extracts is the result of the effect of the diluent on the toxin rather than an increase in the activity of the toxin or any adsorption of the toxin by the tissues. The toxin deteriorates more when diluted with saline than with infusion-broth. Halter's work[8] suggests that oxidation may be the explanation for the deterioration of tetanal toxin in sodium-chloride solution. There is nothing, however, in these experiments to indicate the process of deterioration of staphylococcal toxin.

The amount of necrosis produced in the rabbit's skin by staphylococcal toxin* diluted with physiological saline and diluted with toxin heated at 100°C for 2 hours was studied. This amount of heat is sufficient to destroy the necrotizing properties in this toxin. The following dilutions of toxin in both diluents were injected endermally in 2 animals: 10.0, 6.66, 5.0, 3.33, 2.5, 2.0, and 1.4%. The areas of necrosis were measured 4 days after the toxin was injected. Fig. 1 shows the size of the different necrotic areas. It is evident from this study that staphylococcal toxin deteriorates faster in saline dilution than when diluted with heated toxin. A 3.33% solution of toxin failed to produce necrosis, while a 1.4% solution of the same toxin diluted with heated toxin produced necrosis in the same rabbit. This experiment also shows that the areas of necrosis are not proportional to the amount of toxin injected. The size of the areas of

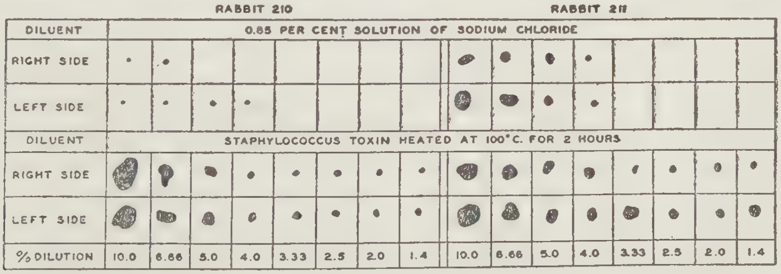

FIG. 1.

Areas of cutaneous necrosis 4 days after the endermal injection of staphylococcal toxin diluted with saline, and heated staphylococcal toxin.

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[8] Halter, K., *Hyg. Infektionskr.*, 1936, **118**, 246.

* Supplied through the courtesy of Lederle Laboratories.

necrosis apparently is influenced by the extent of diffusion of the toxin.

This study of the effect of rabbit tissues on staphylococcal toxin shows that the toxicity decreases in the presence of some of these tissues. The apparent tissue-adsorption may be due largely to the degree of attenuation attained in the presence of different diluents. These *in vitro* observations with staphylococcal toxin are interesting in view of the interpretation of similar experiments of others with tetanus toxin.

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### 10282 P

## Studies on the Chemistry and Immunology of Ragweed Pollen Proteins.

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The opinion has been held for some time in our laboratories that many allergens responsible for atopic symptoms have present two types of protein molecules. An extended study of these allergens has led us to the conclusion that there are usually present representatives of the larger variety, loosely typified by crystallizable egg albumin and also representatives of a type of protein molecule much smaller, approaching the size of the peptone group. In the work of the staff of the Allergy Clinic on the preparation of purified proteins from the various foods and environmental materials, it was found that the more these proteins were purified by repeated precipitation and subsequent dialysis, the less skin reactive they became. Some of them lost practically all of this property. Other investigators have recorded this observation. The usual explanation that has been offered is that the protein has been denatured in the process of purification. This explanation is not valid in the light of experimental evidence in connection with crystallizable egg albumin secured by Cole and Aaronson[1] in this laboratory. They found that with each successive crystallization the skin reaction diminished in certain types of egg sensitive cases until with the sixth crystallization this reaction was quite feeble. The precipitinogenic power of the albumin remained unaltered throughout all the crystallization processes.

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[1] Cole, A. G., and Aaronson, A. L., personal communication.

From this we conclude that the antigenicity of the crystallizable egg albumin was not modified and that the skin reacting substance, either in chemical combination or adsorbed on the albumin, was released during the crystallization processes.

This study was undertaken to determine whether a similar condition existed in the protein content of pollen.

Equal parts of giant and short ragweed pollen were defatted with ether and extracted with distilled water. Rabbits were injected until a serum of a high precipitin titer was obtained against this pollen extract.

Portions of the pollen extract were acidified with various buffers. The precipitates that occurred reached the maximum at a pH between 3.8 and 4.2, usually at 4.0 with a definite diminution in the total nitrogen content of the solution. An additional slight precipitate occurred on heating, reaching its maximum at a temperature between 55 and 60°C. No further precipitate occurred even if this final filtrate was boiled for an hour.

The filtrates from pollen extracts, which had been adjusted to various hydrogen ion concentrations, when brought back to a pH of 6 showed progressive diminutions of precipitin reaction with pollen antiserum as the hydrogen ion was increased, with a maximum decrease at pH 4. The filtrates from the experiment at pH 4 when heated at 60°C for one hour and brought back to a pH of 6 gave no reaction whatever with the pollen antiserum. The precipitates obtained by acidification of the original pollen extract were partly soluble in 0.5% sodium carbonate. The solutions of these precipitates gave definite precipitin reactions with the pollen antiserum.

The physico-chemical properties of the proteins precipitated at pH 3.8-4.2 at 60°C are those that are associated with the larger protein molecules—relative ease of precipitation, quantitative absorption with aluminium cream, very little diffusibility through a permeable membrane such as collodion, and marked viscosity of the solution. On the other hand, the protein fraction remaining in solution has the general characteristic of the smaller protein molecule with more difficulty of precipitation from solution (it is impossible to precipitate quantitatively the skin reacting protein with aluminium cream), moderate power of diffusion through collodion membranes, and less viscosity of the solution.

Skin tests were done on ragweed sensitive patients with the original untreated pollen extracts and the filtrate from which all precipitatable material had been removed at a pH of 3.8 and heating. The filtrate obtained was brought back to a pH of 6.0 to prevent nonspecific irritation. Scarifications were made with a special instrument which produces superficial cuts of practically the same depth and length. The filtrate gave practically undiminished skin tests when compared with an original untreated pollen solution of equivalent nitrogen content. Nor was the skin reaction diminished in the filtrate which had been boiled for an hour. The skin tests were specific for ragweed pollen.

Twelve ragweed sensitive patients were treated preseasonally with the filtrate from which all precipitable material had been removed. None of these had any relief. During the past season a few patients were treated with the filtrate to which gelatin had been added. Some evidence of success was present. However, the number of cases treated was too small and the amount of treatment not sufficient to permit definite conclusions.

*Summary.* The protein of ragweed pollen has been separated into fractions. The one fraction has relatively large molecular size and is responsible for the precipitin reaction with pollen antiserum. The other fraction is made up of proteins of relatively small molecular size and is the skin reactive protein of pollen. This last observation is similar to that of Benjamins and his collaborators,[2] who in their work on grass pollen, came to the conclusion that the skin reacting substance is of small molecular size. Our results differ from those obtained by Harley.[3] He concludes that in timothy pollen the skin reactive fraction consists of proteins of the albumin type. We failed to produce hyposensitization of ragweed sensitive patients by the use of the fraction containing small protein molecules. There is an indication that it may be possible to do so when it is used in connection with large protein molecules. Benjamins and his collaborators found that the skin reactivity of the small protein molecule from grass pollen could be enhanced by the addition of protein of large molecular size. The immunizing properties of the fraction consisting of the larger protein molecules are now being studied.

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[2] Benjamins, C. E., von Dishoeck, H. A. E., and German, J. L. M., *J. Allergy*, 1935, **6**, 335.

[3] Harley, David, *Brit. Exp. Path. J.*, 1937, **18**, 469.

### 10283

# Anticatalase Activity of Sulfanilamide and Related Compounds. II. Relation to Growth Inhibition in Pneumococcus.

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In a previous publication[1] we demonstrated the anticatalase property of sulfanilamide and showed that this attribute is markedly increased by the exposure of dilute solutions to ultraviolet light. It was suggested that anticatalase activity may play an important rôle in the therapeutic action of sulfanilamide against such organisms as the streptococcus and pneumococcus, a theory originally promulgated by Locke.[2]

The hypothetical mechanism may be stated as follows: The growing bacterial cell has the power to convert sulfanilamide, presumably through mild oxidation, into a derivative which is a highly active anticatalase.* This reaction produces an accumulation of anticatalase in the immediate vicinity of the cell. The streptococcus and pneumococcus, being active producers of hydrogen peroxide, are able to grow only so long as the peroxide concentration can be kept below a critical level by outward diffusion or destruction. Usually this is accomplished by catalase of which there is ample reserve in the body and in cultures containing blood. However, in the presence of anticatalase, inactivation of catalase takes place in the zone immediately adjacent to the cell with resultant accumulation of hydrogen peroxide to toxic levels. The feasibility of this explanation is enhanced if we recall that catalase must diffuse into the reaction-zone from the outside environment, whereas anticatalase and peroxide, produced by the cell itself, are already present in the critical zone in their maximal concentrations.

This report presents experimental evidence in support of the foregoing hypothesis.

Ten cc of a 0.2% glucose broth were placed in each of a series of 50 cc Ehrlenmeyer flasks. This amount gave a surface-volume ratio of approximately 1.6.[3] Catalase was supplied through addition of 5 x $10^{-5}$ cc of hemolyzed whole rabbit-blood. This amount consistently gave the most clear-cut results with the particular samples of blood and broth used. Additions of as much as 5 x $10^{-4}$ cc prevented determinable accumulations of peroxide in the medium obscuring recognition of the much higher concentrations probably existing within the reaction-zone.

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[1] Main, E. R., Shinn, L. E., and Mellon, R. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 272.

[2] Locke, A. P., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1938, in press.

* This might be presumed to be the hydroxylamine derivative or even hydroxylamine itself. In this connection we have succeeded in showing by comparative titrations using hydroxylamine and sulfanilamide that if only 2% of the 8 mg% sulfanilamide were converted to hydroxylamine it would produce an anticatalase effect comparable to that obtained on full radiation by ultraviolet light.

Sulfanilamide, as a sterile 500 mg % solution, was added to half the flasks to a final concentration of 8 mg %. All cultures were seeded with a 0.05 cc of an 18-hour broth culture of Type I pneumococcus subjected to prior mouse-passage. The flasks were incubated at 37°C and tested at intervals during the period from 2-6 hours for growth and peroxide-content.

Since the presence of sulfanilamide had been found to encourage chain-formation, it was considered more accurate to estimate growth turbidimetrically against barium sulfate standards rather than by plate-count. Hydrogen peroxide was estimated by adding to 1 cc of the culture a small piece of raw potato as a source of peroxidase, followed by 1 drop of a fresh 1% solution of *o*-tolidine in glacial acetic acid. This reagent develops an intense blue color in the presence of peroxide and is more sensitive that the commonly used benzidine. Concentrations approaching 0.0003% of hydrogen peroxide in broth can be detected.

With the technic employed, detectable peroxide always appeared between 2 and 3 hours in both sulfanilamide- and control-cultures. From the third hour, retardation in growth was apparent in the cultures containing sulfanilamide. The amount of peroxide accumulated in these cultures continued to increase, although the growth, as compared with that of the controls, was becoming increasingly less. Thus the amount of peroxide per unit of bacterial substance became progressively greater in the presence of sulfanilamide. The peroxide-concentrations attained at the 5-hour period in the cultures containing sulfanilamide were of the same general magnitude as is required to cause a corresponding inhibition of growth when added to a similar culture without sulfanilamide (0.001-0.003%).

Because of the limited reproducibility of absolute values inherent in this type of study, attention must be directed to reproducibility of trend. The typically differing trends of peroxide-accumulation and growth in cultures with and without sulfanilamide are shown in Fig. 1. In (A) are shown the peroxide-concentrations reached in

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[3] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, **39**, 275.

3 comparable series,† plotted as the differences between concentration found in the culture containing sulfanilamide and that found in the control culture containing no sulfanilamide. (B) shows a similar plotting of difference in population in the same cultures. The significant portion of the curves begins at about the fourth hour. After this point is reached, the peroxide- and population-curves diverge.

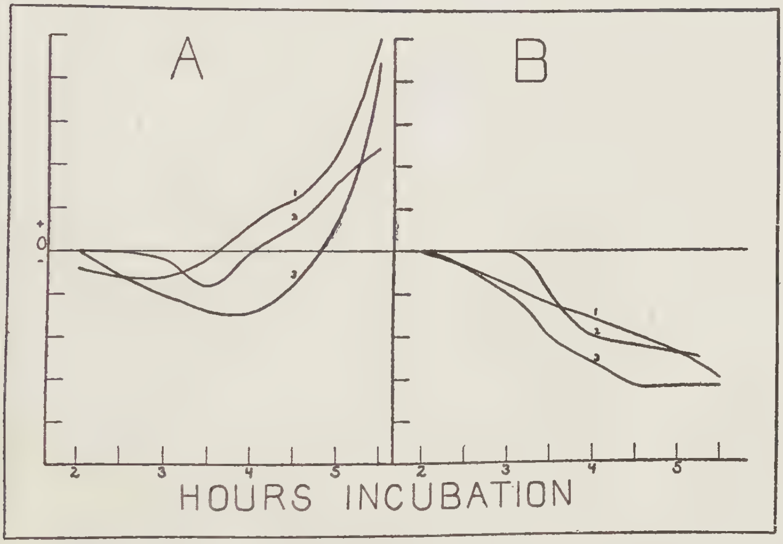

FIG. 1.

A. Curves representing the preponderance of peroxide in cultures containing sulfanilamide over peroxide in cultures not containing sulfanilamide.

B. Curves representing the preponderance (negative) in population of cultures containing sulfanilamide over that of cultures not containing sulfanilamide.

Curves 1, 2, and 3 refer to 3 separate comparable experiments.

No values are attached to the ordinates because the limited reproducibility of absolute magnitudes deprives them of real significance. The point to be illustrated is the direction of the trend.

Theoretically, such divergence would be expected. The presence of sulfanilamide permits the accumulation of peroxide, which, through its toxic effect, retards growth of the organisms. The depression in growth would, in turn, be expected to lower the rate of peroxide-formation. However, peroxide accumulates in spite of the reduced growth, in amounts even larger than those associated with a normal growth-rate.

The negative value assumed by the peroxide-difference curves during the early period is possibly due to the fact that the points defined are by necessity limited to estimations of the amount of peroxide that has diffused out into the surrounding medium rather than the amount present in the effective zone immediately about the organism. If a diffusion-lag of approximately one hour is assumed, the time of average upturn of the peroxide-curves corresponds closely to the point of beginning fall in the population-curves. This would relate the depressed values for peroxide to the very early growth-period regarding which little information is available other than that stimulation by sulfanilamide during this period takes place.[4]

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† The cultures, in these series, received additions of 5 x $10^{-5}$ cc of blood. Series both without added catalase and with catalase added in higher amount gave results corroborative in direction but of magnitudes of difference less satisfactory for graphic representation.

Regardless of the interpretation placed on this feature, it is nevertheless clear that under the conditions of the experiment growth is inhibited by sulfanilamide and this inhibition is accompanied by an increase in peroxide as compared with control cultures. This we consider to be supportive evidence for the theory that the mechanism of sulfanilamide action is in part through the inactivation of catalase.

The reported strong activity of sulfanilamide against highly virulent strains as compared to those of low virulence may find explanation in the anticatalase-hypothesis. Highly virulent strains, particularly in the streptococci, are usually heavily encapsulated. In the pneumococcus, loss of capsule means loss of virulence. Assuming that the conception of an anticatalase-mechanism is correct, it is not inconceivable that the extent and nature of the capsule about the organism may play a definite rôle. Any factor that restrains the inward diffusion of catalase and the outward diffusion of peroxide and anticatalase should necessarily increase the degree of peroxide-accumulation. The capsule may function in this way by creating a stabilized zone in which diffusion-rates are reduced.

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[4] Locke, A. P., personal communication.

**All preliminary manuscripts are indicated by the letter P after the number of the article**

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